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(54) Title: METHOD OF MODIFYING PLANT MORPHOLOGY, BIOCHEMISTRY AND PHYSIOLOGY II

(57) Abstract

The present invention provides a method of modifying one or more morphological, biochemical and physiological properties or characteristics in a plant, said method comprising expressing a cyclin protein, in particular cyclin B, in the plant, operably under the control of a regulatable promoter sequence.

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METHOD OF MODIFYING PLANT MORPHOLOGY, BIOCHEMISTRY AND PHYSIOLOGY II

FIELD OF THE INVENTION

The present invention relates generally to a method of modifying plant morphological, biochemical and physiological properties or characteristics, such as one or more environmental adaptive responses and/or developmental processes, said method comprising expressing a cell cycle control protein, in particular cyclin B, in the plant, operably under the control of a regulatable promoter sequence. Preferably, the characteristics modified by the present invention are cytokinin-mediated and/or gibberellin-mediated characteristics. The present invention extends to gene constructs which are useful for performing the inventive method and to transgenic plants produced therewith having altered morphological and/or biochemical and/or physiological properties compared to their otherwise isogenic counterparts.

GENERAL

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Throughout this specification, unless the context requires otherwise the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

As used herein, the term "derived from" shall be taken to indicate that a particular

integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

BACKGROUND TO THE INVENTION

Development and environmental adaptation are highly regulated processes in plants. These processes are not cell-autonomous but rather involve extensive communication between different parts of the plant. Amongst the most important mobile signals involved in this long-distance communication are plant hormones such as auxins, cytokinins, abscisic acid, gibberellins, and ethylene. Other signals, so far not defined as plant hormones, include salicyclic acid, jasmonic acid and brassinosteroids.

There are plethora of data showing that the external application of plant hormones has profound effects on development, metabolism and environmental fitness. For example, the external application of cytokinins produces a variety of morphological, biochemical and physiological effects in plants, including the stimulation of organogenesis, shoot initiation from callus cultures, release of lateral buds from apical dominance, dwarf growth, alteration of source/sink relationships, stimulation of pigment synthesis, inhibition of root growth, and delay of senescence. Additionally, exogenous cytokinin application following anthesis in cereals enhances grain set and yield and the phase of nuclear and cell division in the developing endosperm of cereal grains is accompanied by a peak of cytokinin concentration, suggesting a role for cytokinins in grain development in cereals (Herzog, 1980; Morgan *et al.*, 1983). Cytokinins have also been implicated in promoting the initiation of tuber formation in potato (International Patent Publication No. WO 93/07272) and in improving the resistance of potato plants to insects (United States Patent No. 5, 496, 732) and in inducing male sterility and partial female sterility in tobacco plants (European Patent No. EP-A-334,383).

The effect of cytokinin on plant development and morphology may be attributed, at least in part, to modified biochemistry of the plant, such as a modification to the source/sink relationship in the plant or plant part.

Attempts to modify plant cytokinin-mediated and/or gibberellin-mediated growth and developmental responses employ the exogenous application of cytokinins and/or gibberellins respectively. Such approaches are costly and produce undesirable pleiotropic side-effects on the plant tissue.

Other approaches to modifying plant cytokinin-mediated growth and developmental responses employ the ectopic expression of an introduced bacterial isopentenyladenosine transferase (IPT) gene (International Patent Publication No. WO 93/07272; United States Patent No. 5, 496, 732; United States Patent No. 5, 689, 042) under the control of a strong constitutive promoter sequence, developmentally-regulated promoter sequence or hormonally-inducible promoter sequence. Alternatively, plant cytokinin-mediated growth and developmental responses have been modified by the ectopic expression of the *Agrobacterium rhizogenes RolC* gene (European Patent No. EP-A-334,383). These approaches also produce undesirable side-effects in the plant and, even in cases where *ipt* or *rolC* is expressed under the control of tissue-specific promoters, these side-effects are observed in other tissues, presumably because the cytokinin is transported readily between cells and tissues of the plant.

SUMMARY OF THE INVENTION

In work leading to the present invention, the present inventors sought to develop a method of producing specific targeted modifications to plant morphology, biochemistry and physiology, in particular specific target modifications to cytokinin-mediated and gibberellin-mediated plant growth and development, thereby avoiding the problem of pleiotropy associated with the prior art.

Surprisingly, the inventors discovered that the targeted ectopic expression of a cell cycle control protein such as cyclin B in particular cells, tissues or organs of the plant produces localised specific modifications to cytokinin-mediated cellular metabolism and cell fate compared to otherwise isogenic non-transformed plants.

More particularly, the inventors have discovered that the G2 phase of the cell cycle in

plants can be shortened by the ectopic expression of the alfalfa CycMs2 protein therein. This shortening of the G2 phase results in altered cell fate and sink/source relationships in plants expressing CycMs2, thereby mimicking many of the cytokinin-mediated and/or gibberellin-mediated developmental and biochemical processes which can be induced by the exogenous application of phytohormones, without the undesirable side-effects of the prior art.

The shortening of the G2 phase by ectopic expression of alfalfa CycMs2 in tobacco is shown in Example 3. Whilst not being bound by any theory or mode of action, it is likely that the ectopic expression of a cyclin B such as alfalfa CycMs2, or ectopic expression of a cyclin B-type protein, in plants, advances cell division by advancing the entry of cells into mitosis, which modifies cellular metabolism via a mechanism that involves modifications to the partitioning of carbon and/or the activities of one or more enzymes involved in carbon partitioning (eg. invertase) and/or the levels of regulatory molecules such as sucrose, ATP, ADP and inorganic orthophosphate. Because cell fate is in some way related to the cell cycle, the altered cellular metabolism which occurs is able to modify cell fate. For example, roots can be regenerated from calli in culture when the cells exit the cell cycle in G2 phase, while shoot formation is dependent upon an exit from the cell cycle in the G1 phase, such that the formation of roots versus shoots depends upon the phase (G1 or G2) at which cells exit the cell cycle. This proposed mechanism may explain the inhibition of root regeneration observed by the present inventors (*see* Example 4) when the G2/M transition and/or the duration of the G2 phase is shortened by ectopic expression of CycMs2.

Cyclin B is an intracellular protein, which, unlike exogenously-applied cytokinins or cytokinins produced by ectopic expression of *ipt* or *rolC* genes, will only exert a localised effect at the site of protein synthesis. This observation has led the present inventors to develop methods for controlled expression of alfalfa CycMs2 in particular cells, tissues and organs of plants, for the purposes of modifying cytokinin-mediated plant morphology and/or biochemistry and/or physiology, and to facilitate the selection of specific cells, tissues and organs which exhibit cytokinin-mediated morphological characteristics and/or biochemical characteristics and/or physiological characteristics.

Accordingly, one aspect of the invention provides a method of modifying cell fate and/or plant development and/or plant morphology and/or biochemistry and/or physiology comprising expressing in particular cells, tissues or organs of a plant, a genetic sequence encoding a cell cycle control protein, and, in particular a cyclin protein, operably in connection with a plant-operable promoter sequence.

Expression of the cell cycle control protein is preferably carried out by introducing an isolated nucleic acid molecule comprising the protein-encoding nucleotide sequence into a cell, tissue or organ of the plant, regenerating plant tissue or whole plants therefrom and then culturing those plant parts or whole plants under conditions suitable for activity of the promoter sequence to which said nucleotide sequence is operably connected.

Preferably, the genetic sequence encoding the cyclin protein is placed operably under the control of a plant-expressible promoter sequence selected from the list comprising strong constitutive promoter sequences, cell-specific promoter sequences, tissue-specific promoter sequences, organ-specific promoter sequences, cell-cycle-specific promoter sequences, and inducible promoter sequences (both pathogen-inducible and environmentally-inducible promoters are contemplated herein). The present invention further encompasses the use of a promoter sequence in a gene construct wherein an excisable genetic element is inserted into said construct so as to inactivate expression of the cyclin protein during transformation and regeneration steps. According to this embodiment, excision of the excisable genetic element in the regenerated plant or progeny derived therefrom facilitates ectopic expression of the cyclin protein. Methods to induce excision of such genetic elements are known to those skilled in the art. The excisable genetic element may be an autonomous or non-autonomous excisable genetic element.

In a particularly preferred embodiment of the invention, the cyclin protein is a cyclin B protein, and, more particularly, the alfalfa CycMs2 protein, or a biologically-active homologue, analogue or derivative thereof. The present invention clearly contemplates

the use of functional homologues of cyclin B proteins. Accordingly, the present invention is not limited in application to the use of nucleotide sequences encoding the alfalfa CycMs2 protein.

The present invention clearly extends to the use of modified cyclin B proteins, or substrates of a cyclin B protein, or modified substrates of a cyclin B protein, that produce the same effects in respect of the present invention as can be produced using a cyclin B protein, in particular the alfalfa CycMs2 protein described herein.

The ectopic expression of a cyclin protein or a homologue, analofue or derivative thereof in a plant can produce a range of desirable phenotypes in plants, such as, for example, by modifying one or more morphological, biochemical, or physiological characteristics as follows: (i) modifying the length of the G2 phase of the cell cycle of a plant; (ii) modifying the G2/M phase transition of a plant cell; (iii) modification of the initiation, promotion, stimulation or enhancement of cell division; (iv) modification of the initiation, promotion, stimulation or enhancement of DNA replication;(v) modification of the initiation, promotion, stimulation or enhancement of seed set and/or size and/or development; (vi) modification of the initiation, promotion, stimulation or enhancement of tuber formation; (vii) modification of the initiation, promotion, stimulation or enhancement of shoot initiation and/or development; (viii) modification of the initiation, promotion, stimulation or enhancement of root initiation and/or development; (ix) modification of the initiation, promotion, stimulation or enhancement of lateral root initiation and/or development; (x) modification of the initiation, promotion, stimulation or enhancement of nodule formation and/or nodule function; (xi) modification of the initiation, promotion, stimulation or enhancement of bushiness of the plant; (xii) modification of the initiation, promotion, stimulation or enhancement of dwarfism in the plant; (xiii) modification of the initiation, promotion, stimulation or enhancement of pigment synthesis; (xiv) modification of source/sink relationships; (xv) modification of carbon partitioning in the plant; (xvi) modification of the initiation, promotion, stimulation or enhancement of senescence; and (xvii) modification of stem thickness and/or strength characteristics and/or wind-resistance of the stem.

As used herein, unless specifically stated otherwise, the term "modification of the initiation, promotion, stimulation or enhancement" in relation to a specified integer shall be taken as a clear indication that the integer is capable of being enhanced, increased, stimulated, or promoted, or alternatively, decreased, delayed, repressed, or inhibited.

In a preferred embodiment of the present invention, a cyclin B protein or a homologue, analogue or derivative thereof, such as, for example, a modified substrate of cyclin B that mimics the effect of cyclin B is expressed operably under the control of a regulatable promoter that is expressible in a plant cell, to shorten the duration of the G2 phase and/or to shorten the G2/M phase transition of said cell.

In another preferred embodiment of the present invention,a cyclin B protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B is expressed operably under the control of a regulatable promoter that is expressible in a plant cell, tissue or organ to modify cell fate and/or plant development.

Other preferred embodiments of the invention relate to the effect(s) of cytokinins and/or gibberellins on plant metabolism. With respect to such hormone-mediated effects, the present invention clearly contemplates the broad application of the inventive method to the modification of a range of cellular processes mediated by cytokinins and/or gibberellins, including but not limited to cellular development and/or cell fate; the advancement of cell division; the initiation, promotion, stimulation or enhancement of seed development and/or tuber formation and/or shoot initiation and/or bushiness and/or dwarfism and/or pigment synthesis, and/or the modification of source/sink relationships, and/or the modification of root growth and/or the inhibition of root apical dominance and/or the delay of senescence.

Accordingly, in another preferred embodiment of the present invention, a cyclin B protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B is expressed operably under the control of a regulatable promoter that is expressible in a plant cell, tissue or organ to modify

carbon partitioning between the cells, tissues, or organs of plants.

In another preferred embodiment of the present invention, a cyclin B protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B is expressed operably under the control of a regulatable promoter that is expressible in a plant cell, tissue or organ to modify sink/source relationships in the plant, and, in particular, in the seed of a plant. For modification of sink/source relationships in the seed of a plant, the cyclin protein is preferably expressed under the control of a promoter sequence that is operable in the endosperm of the seed, in which case the seed produced exhibit enhanced grain filling and higher levels of starch in the dried seed than the seed of otherwise isogenic plants.

In another preferred embodiment of the present invention, a cyclin B protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B is expressed operably under the control of a promoter derived from a seed-expressible gene, to increase seed production in plants, in particular to increase seed set, seed size, and seed yield. More preferably, the promoter is operable in the endosperm of the seed or in the storage cotyledon, in which cases the combination of the cell cycle-control protein and endosperm-expressible or cotyledon-expressible promoter provides the additional advantage of increasing the grain size and grain yield of the plant.

In still another preferred embodiment of the present invention, a cyclin B or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B is expressed operably under the control of a promoter derived from a leaf-expressible gene, to prevent or delay or otherwise reduce leaf chlorosis and/or leaf necrosis and/or leaf sensecence.

In yet another preferred embodiment of the present invention, a cyclin B protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B is expressed operably under the control of a promoter derived from a meristem-expressible gene or a shoot-expressible gene or root-

expressible gene, to reduce bushiness of the plant. In a related embodiment, such promoter/cyclin B combinations are used to reduce root apical dominance in plants.

In another preferred embodiment of the present invention, a cyclin B protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B is expressed operably under the control of a promoter derived from a stem-expressible gene, to increase the strength and thickness of a plant stem to confer improved stability and wind-resistance on the plant.

In another preferred embodiment of the present invention, a cyclin B protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B is expressed in a tuber-forming plant operably under the control of a promoter derived from a stem-expressible gene or tuber-expressible gene, to increase improve tuber production in the plant.

In another embodiment of the present invention, a cyclin B protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B is expressed in a tree crop plant such as, but not limited to, *Eucalyptus spp.* or *Populus spp.*, operably under the control of a promoter derived from a gene that is expressed in vascular tissue and/or cambium cells, to increase lignin content therein. Without being bound by any theory or mode of action, the ectopic expression of cyclin B under control of a promoter that is operable in vascular tissue and preferably, in cambial cells, will produce thick-stemmed plants and a higher ratio of vascular tissue-to-pith cells within the stem, thereby resulting in more lignin production. Within the vascular tissue, cambial cells contain the highest levels of auxins and are therefore the preferential tissue for cyclin B overproduction.

In a further preferred embodiment of the present invention, a cyclin B protein or a homologue analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B is expressed under the control of a promoter that is operative in meristem tissue of grain crops, to stimulate cell division in the intercalary meristem of the youngest stem internode and produce greater elongation of the stem

and/or to generate a more extensive photosynthetic canopy.

A second aspect of the invention provides a gene construct or vector comprising a nucleotide sequence that encodes a cyclin protein, such as, for example, a cyclin B protein, and in particular, the alfalfa CycMs2 protein, or a homologue, analogue, or derivative thereof, placed operably under the control of a plant-expressible promoter sequence selected from the group consisting of:

(i) a plant-expressible cell-specific promoter sequence;

(ii) a plant-expressible tissue-specific promoter sequence;

(iii) a plant-expressible organ-specific promoter sequence;

(iv) a plant expressible inducible promoter sequence;

(v) a plant-expressible cell cycle specific gene promoter sequence;

(vi) a plant-expressible constitutive promoter sequence, wherein the nucleotide sequence encoding said cyclin protein, and the plant-expressible constitutive promoter sequence, are integrated into an excisable genetic element; and

(vii) a plant-expressible constitutive promoter sequence, wherein the nucleotide sequence encoding saidcyclin protein and said promoter sequence are such that expression of said substrate or modified substrate is capable of being modulated by an excisable genetic element.

Preferably, the gene construct or vector according to this aspect of the invention is suitable for expression in a plant cell, tissue, organ or whole plant and more preferably, the subject gene construct or vector is suitable for introduction into and maintenance in a plant cell, tissue, organ or whole plant.

A third aspect of the invention provides a plant cell, tissue, organ or whole plant that has been transformed or transfected with an isolated nucleic acid molecule that comprises a nucleotide sequence which encodes a cyclin protein, wherein the expression of said nucleotide sequence is placed operably under the control of a plant-expressible cell-specific promoter sequence, plant-expressible tissue-specific promoter sequence, a plant-expressible organ-specific promoter sequence, a plant-expressible

cell cycle gene specific promoter, or alternatively, a plant-expressible constitutive promoter sequence such that said plant-expressible constitutive promoter sequence and said nucleotide sequence encoding a cell cycle control protein are integrated into an excisable genetic element.

This aspect of the invention extends to plant propagules and plant parts that contain the introduced nucleic acid molecule and have the potential to reproduce one or more of the phenotypes of the primary transformants/transfectants, either by inducing gene expression directly therein or by the application of standard breeding or recombinant technologies.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1-1 is a copy of a photographic representation of a northern blot hybridisation showing the expression of alfalfa *CycMs2-HA* mRNA in the leaves of six transgenic tobacco lines from a tetracyclin-regulatable promoter sequence, either in the absence (-) or presence (+) of 1 mg/dm ^{3} Cl-tetracycline. The positions of CycMs2-HA mRNA and a control mRNA (pCNT6) are indicated.

Figure 1-2 is a copy of a photographic representation of a northern blot hybridisation showing the time-dependent accumulation of alfalfa *CycMs2-HA* mRNA in a suspension culture initiated from line 2 (Figure 1-1) on 2,4-D-containing medium and subsequently incubated in medium containing 1 mg/dm ^{3} Cl-tetracycline. The positions of CycMs2-HA mRNA and a control mRNA (CycM) are indicated. Numbers at the top of the figure indicate elapsed time (hours) following addition of tetracycline.

Figure 1-3 is a copy of a photographic representation showing the tetracycline concentration-dependent expression of CycMs2-HA fusion protein in cultured tobacco cells. In the top panel, a western blot of total cellular extracts was probed with anti-HA antibody. In the middle panel, a western blot of immunopurified CDK complexes were probed with anti-HA antibodies. In the lower panel, CDK complexes were purified by binding to the p13 ^{suc1} protein. The position of the CycMs2-HA fusion protein is indicated in the upper and middle panels, whilst the position of total p13 ^{suc1} protein-

binding CDK fraction is indicated in the lower panel (p13 ^{suc1}).

Figure 2 is a copy of a photographic representation of an immunofluorescence micrograph probed with anti-HA antibody, showing the expression of the CycMs2-HA fusion protein in the nuclei of transgenic tobacco cells following induction by tetracycline. As a control, cells were stained using DAPI to identify the nuclear DNA.

Figure 3 is a copy of a photographic representation showing the histone-kinase activities of CycMs2-HA-associated CDKs (i.e. CycMs2-HA) and p13 ^{suc1} protein-bound CDKs (p13 ^{suc1}) in total cellular extracts, and in the nuclear and cytoplasmic fractions of tobacco cells expressing alfalfa CycMs2 as a fusion protein with a haemaglutinin epitope tag (HA).

Figure 4-1 is a copy of a graphical representation showing the percentage of aphidicolin-released tobacco cells entering mitosis following tetracycline induction of *CycMs2* gene expression (+) or in the absence of tetracycline induction (-), as a function of time.

Figure 4-2 is a copy of a graphical representation showing the percentage of microtubule structures in aphidicolin-released tobacco cells following tetracycline induction of *CycMs2* gene expression (+) or in the absence of tetracycline induction (-), as a function of time.

Figure 4-3 is a copy of a graphical representation showing the percentage of aphidicolin-released tobacco cells entering mitosis following tetracycline induction of *CycMs2* gene expression (+) or in the absence of tetracycline induction (-), as determined by flow cytometry and expressed as a function of time.

Figure 4-4 is a copy of a graphical representation showing the shortened duration of the G2 phase in aphidicolin-released tobacco cells following tetracycline induction of *CycMs2* gene expression (+) compared to isogenic cells incubated in media lacking tetracycline (-).

Figure 5 is a copy of a photographic representation of a northern blot hybridisation showing the expression of *CycMs2* mRNA in G2 phase cells ectopically expressing the CycMs2-HA fusion protein, compared to the levels of histone *H4* mRNA; the endogenous tobacco *CycM* mRNA; and the control pCNT6. Cells expressing the CycMs2-HA fusion protein were incubated in the presence of tetracycline (+ tetracycline), whilst cells not expressing this fusion protein were incubated in the absence of tetracycline (- tetracycline).

Figure 6 is a copy of a photographic representation showing the inhibition of root regeneration from tobacco leaves of transgenic lines that ectopically express alfalfa CycMs2 mitotic cyclin.

Figure 7 is a copy of a graphical representation of a flow cytometric analysis of the DNA contents in tetracyclin-treated (+tet) and untreated (-tet) tobacco leaves or leaf discs, in the absence of cytokinin and auxin (0/0), or treated with different cytokinin:auxin ratios (i.e. 0.5/0.1; 0.1/0.5) showing the higher percentage of cells with G2 DNA content in leaf cells derived from wild-type plants treated with high auxin:cytokinin [0.5/0.1 (root)], compared to leaf cells derived from wild-type plants treated with low auxin:cytokinin [0.1/0.5 (shoot)] or on equivalent levels of cytokinins and auxins [0.5/0.5(callus)].

Figure 8 is a copy of a schematic representation of non-limiting cell cycle model of shoot/root regeneration.

Figure 9 is a copy of a photographic representation showing the ectopic expression of CycMs2 retards plant growth and mimics cytokinin effect in dark-growth seedlings. TM 100 2/5 are seedlings transformed with CycMs2. Bin Hyg are transformants with the control plasmid pBin-HygTX.

Figure 10 is a copy of a photographic representation of a Epifluorescence microphotograph of GFP fluorescence (A); and DIC phase contrast image of the cell (B) transformed with a CycMs2-GFP fusion.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

One aspect of the present invention provides a method of modifying cell fate and/or plant development and/or plant morphology and/or biochemistry and/or physiology comprising expressing in particular cells, tissues or organs of a plant, comprising expressing in said cells, tissues or organs a genetic sequence encoding a cell cycle control protein operably under the control of a regulatable promoter sequence selected from the list comprising cell-specific promoter sequences, tissue-specific promoter sequences, and organ-specific promoter sequences.

Preferably, the plant morphological, biochemical or physiological characteristic which is modified is a cytokinin-mediated or a gibberellin-mediated characteristic.

The word "modify" or variations such as "modifying" or "modified" as used herein with reference to any specified integer or group of integers shall be taken to indicate that said integer is altered by the performance of one or more steps pertaining to the invention described herein, compared to said integer in the absence of such performance.

Accordingly, by "cell fate and/or plant development and/or plant morphology and/or biochemistry and/or physiology" is meant that one or more developmental and/or morphological and/or biochemical and/or physiological characteristics of a plant is altered by the performance of one or more steps pertaining to the invention described herein.

"Cell fate" refers to the cell-type or cellular characteristics of a particular cell that are produced during plant development or a cellular process therefor, in particular during the cell cycle or as a consequence of a cell cycle process. Our results presented herein indicate that increased mitotic cyclin expression produces a shortened G2-phase in plant cells. It is likely that mitotic cyclin overexpression has a cytokinin-like effect, evidenced by our finding that mitotic cyclin overproduction results in the suppression of root formation.

The shape of a plant is the result of the polarity of auxin and cytokinin. Auxins are produced in the shoot tips where they maintain the shoot meristem. They suppress further shoot formation (shoot apical dominance) and induce root formation. Cytokinins are produced in the root tips where they maintain the root meristem. They suppress further root formation and induce shoot formation. Thus, root suppression by mitotic cyclin overexpression can be seen as a cytokinin effect. A further finding was that, at a critical period in root formation from leaf disks (two weeks after incubating excised leaf disks on an auxin-containing medium), dividing cell populations giving rise to roots had an extended G2 phase. Tetracycline-induced mitotic cyclin overexpression abolished this extended G2 phase and suppressed root formation, suggesting a link between a long G2 phase and root formation. This is the first time that it has been shown that the experimentally controlled length of a phase of the plant cell cycle dramatically alters the developmental fate of these cells.

The fact that cyclin overexpression throughout the plant affects not only the length of a cell cycle phase but suppresses a specific developmental potency of certain cells such as root cells, indicates that cyclin overexpression mimics a specific effect of a plant hormone and has negative pleiotropic effects, at least in these cells.

Accordingly, a novel strategy for designing novel crops would therefore be to overexpress or to repress the expression of specific cyclins in specific cell, tissue or organ types via the use of appropriate regulatable promoter sequences, in order to modify the pleiotropic effects linked to the specific cyclin, and, in particular to modify those peiotropic effects that are hormone-mediated (such as, for example cytokinin-mediated and/or gibberellin-mediated effects) in the plant. In the case of ectopically-expressed cyclins, the pleiotropic effect of the cyclin in question is enhanced. In the case of repressed expression of cyclins, the pleiotropic effect of the cyclin in question is reversed or suppressed and consequent de-repression of a repressed cyclin, using for example an inducible promoter, induces the formation of organs/cell types/structures/functions that were previously repressed. Persons skilled in the art are aware of means for repressing the expression of genes in plants, for example using antisense, co-suppression, post-transcriptional gene silencing, targetted gene

disruption (i.e. gene-targeting), ribozyme-mediated approaches, chemical mutagenesis, T-DNA-mediated mutagenesis, and transposon-mediated mutagenesis, amongst others. The present invention clearly extends to all such embodiments.

Moreover, induced downregulation of cyclins provides for controlled morphogenesis in the one or the other or both directions. Non-morphogenic cell cultures, such as those overexpressing a mitotic cyclin and a G1 cyclin, are useful for biomass production, particularly when coupled with autotrophy.

"Plant development" or the term "plant developmental characteristic" or similar term shall be taken to mean any cellular process of a plant that is involved in determining the developmental fate of a plant cell, in particular the specific tissue or organ type into which a progenitor cell will develop. Cellular processes relevant to plant development will be known to those skilled in the art. Such processes include, for example, morphogenesis, photomorphogenesis, shoot development, root development, vegetative development, reproductive development, stem elongation, flowering, and regulatory mechanisms involved in determining cell fate, in particular a process or regulatory process involving the cell cycle. For example, in the present context, the inventors have shown that the development of roots from tobacco leaf discs in culture can be inhibited by shortening the G2 phase of the cell cycle.

"Plant morphology" or the term "plant morphological characteristic" or similar term will be understood by those skilled in the art to refer to the external appearance of a plant, including any one or more structural features or combination of structural features thereof. Such structural features include the shape, size, number, position, colour, texture, arrangement, and patternation of any cell, tissue or organ or groups of cells, tissues or organs of a plant, including the root, stem, leaf, shoot, petiole, trichome, flower, petal, stigma, style, stamen, pollen, ovule, seed, embryo, endosperm, seed coat, aleurone, fibre, cambium, wood, heartwood, parenchyma, aerenchyma, sieve element, phloem or vascular tissue, amongst others.

The suppression of organ formation by cyclin overexpression may be used to

specifically design plant architecture or morphology, particularly in crops in which apical dominance is weak (e.g. in fruit trees, fruit-bearing crop plants such as tomato, vegetable crops, or cereals). Shoot apical dominance has been described as a mechanism by which the dominant organs repress growth elsewhere in the plant by acting as an auxin-induced sink for root-derived cytokinins.

"Plant biochemistry" or the term "plant biochemical characteristic" or similar term will be understood by those skilled in the art to refer to the metabolic and catalytic processes of a plant, including primary and secondary metabolism and the products thereof, including any small molecules, macromolecules or chemical compounds, such as but not limited to starches, sugars, proteins, peptides, enzymes, hormones, growth factors, nucleic acid molecules, celluloses, hemicelluloses, calloses, lectins, fibres, pigments such as anthocyanins, vitamins, minerals, micronutrients, or macronutrients, that are produced by plants.

Because most biosynthetic activities of plant cells, such as *in vitro*-cultured cells, are linked to differentiation, such cell cultures may be of interest for fermentation. In a first step, high CDK activity would allow cell mass production, selective downregulation of plant cyclins may derepress morphogenesis and specific biosynthetic activities (e.g. secondary plant metabolite production) may occur.

"Plant physiology" or the term "plant physiological characteristic" or similar term will be understood to refer to the functional processes of a plant, including developmental processes such as growth, expansion and differentiation, sexual development, sexual reproduction, seed set, seed development, grain filling, asexual reproduction, cell division, dormancy, germination, light adaptation, photosynthesis, leaf expansion, fibre production, secondary growth or wood production, amongst others; responses of a plant to externally-applied factors such as metals, chemicals, hormones, growth factors, environment and environmental stress factors (eg. anoxia, hypoxia, high temperature, low temperature, dehydration, light, daylength, flooding, salt, heavy metals, amongst others), including adaptive responses of plants to said externally-applied factors.

In addition to modifying cell fate, regulating the bioactivity of a cyclin protein or a homologue, analogue or derivative thereof in a plant, such as by ectopically expressing said cyclin protein, or by reducing the expression of endogenous cyclin proteins, preferably produces a wide range of desirable phenotypes in the plant, such as, for example, a morphological, biochemical or physiological characteristic selected from the group consisting of:(i) enhanced seed size; (ii) enhanced grain yield; (iii) enhanced stem strength; (iv) enhanced stem thickness; (v) enhanced stem stability; (vi) enhanced wind-resistance of the stem; (vii) enhanced tuber formation; (viii) enhanced tuber development; (ix) increased lignin content; (x) enhanced ploidy of the seed; (xi) enhanced endosperm size; (xii) reduced apical dominance; (xiii) increased bushiness; (xiv) enhanced lateral root formation; (xv) enhanced rate of lateral root production; (xvi) enhanced nitrogen-fixing capability; (xvii) enhanced nodulation or nodule size; (xviii) reduced or delayed leaf chlorosis; (xix) reduced or delayed leaf necrosis; (xx) partial or complete inhibition of the arrest of DNA replication in a plant cell under growth-limiting conditions; (xxi) enhanced endoreplication; (xxii) enhanced endoreduplication, including enhanced endoreduplication in the seed; and (xxiii) enhanced cell expansion.

More preferably, the plant morphological, biochemical or physiological characteristic which can be modified by modifying the bioactive concentration of cyclin protein is a cytokinin-mediated or a gibberellin-mediated characteristic selected from the group consisting of: (i) enhanced stem thickness; (ii) enhanced stem stability; (iii) enhanced wind-resistance of the stem; (iv) enhanced tuber formation; (v) enhanced tuber development; (vi) increased lignin content; (vii) enhanced seed set; (viii) enhanced seed production; (ix) enhanced grain yield; (x) enhanced ploidy of the seed; (xi) enhanced endosperm size; (xii) reduced apical dominance; (xiii) increased bushiness; (xiv) enhanced lateral root formation; (xv) enhanced rate of lateral root production; (xvi) enhanced nitrogen-fixing capability; (xvii) enhanced nodulation or nodule size; (xviii) reduced or delayed leaf chlorosis; (xix) reduced or delayed leaf necrosis; (xx) partial or complete inhibition of the arrest of DNA replication in a plant cell under growth-limiting conditions; (xxi) enhanced endoreplication and/or enhanced endoreduplication; and (xxii) enhanced cell expansion.

In a particularly preferred embodiment of the invention, a genetic sequence encoding a cyclin protein is expressed ectopically in a plant , or a plant part, cell, tissue or organ, to: (i) shorten the G2 phase of the cell cycle; and/or (ii) to shorten the G2/M phase transition of a cell; and/or (iii) to inhibit root regeneration from calli; and/or (iv) to stimulate shoot formation from calli; and/or (v) to promote, stimulate or enhance bushiness of a plant; and/or (vi) to inhibit, delay, or reduce apical dominance in a plant; and/or (vii) to modify source/sink relationships in the plant, and particularly in the seed; and/or (viii) to inhibit, delay, or reduce leaf senescence (chlorosis and/or necrosis); and/or (ix) to enhance endoreplication, endoreduplication, or otherwise modify DNA synthesis by overriding the DNA synthesis checkpoint in a cell.

The word "express" or variations such as "expressing" and "expression" as used herein shall be taken in their broadest context to refer to the transcription of a particular genetic sequence to produce sense or antisense mRNA or the translation of a sense mRNA molecule to produce a peptide, polypeptide, oligopeptide, protein or enzyme molecule. In the case of expression comprising the production of a sense mRNA transcript, the word "express" or variations such as "expressing" and "expression" may also be construed to indicate the combination of transcription and translation processes, with or without subsequent post-translational events which modify the biological activity, cellular or sub-cellular localization, turnover or steady-state level of the peptide, polypeptide, oligopeptide, protein or enzyme molecule.

The term "cell cycle" as used herein shall be taken to include the cyclic biochemical and structural events associated with growth and with division of cells, and in particular with the regulation of the replication of DNA and mitosis. Cell cycle includes phases called: G0 (gap 0), G1 (gap 1), DNA replication (S), G2 (gap 2), and mitosis including cytokinesis (M). Normally these four phases occur sequentially. However, the cell cycle also includes modified cycles such as endomitosis, acytokinesis, polyploidy, polyteny, endopolyploidisation and endoreduplication or endoreplication.

The term "cell cycle interacting protein", "cell cycle protein", or "cell cycle control protein" as denoted herein means a protein which exerts control on or regulates or is

required for the cell cycle or part thereof of a cell, tissue, organ or whole organism and/or DNA replication. It may also be capable of binding to, regulating or being regulated by cyclin dependent kinases or their subunits. The term also includes peptides, polypeptides, fragments, variant, homologues, alleles or precursors (eg preproproteins or preproteins) thereof.

Cell cycle control proteins and their role in regulating the cell cycle of eukaryotic organisms are reviewed in detail by John (1981) and the contributing papers therein (Norbury and Nurse 1992; Nurse 1990; Ormond and Francis 1993) and the contributing papers therein (Doerner *et al.* 1996; Elledge 1996; Francis and Halford 1995; Francis *et al.* 1998; Hirt *et al.* 1991; Mironov *et al.* 1999) which are incorporated herein by way of reference.

The term "cell cycle control gene" refers to any gene or mutant thereof which exerts positive or negative control on, or is required for, chromosomal DNA synthesis, mitosis (preprophase band, nuclear envelope, spindle formation, chromosome condensation, chromosome segregation, formation of new nuclei, formation of phragmoplast, etc) meiosis, cytokinesis, cell growth, or endoreduplication. The term "cell cycle control gene" also includes any and all genes that exert control on a cell cycle protein as hereinbefore defined, including any homologues of CDKs, cyclins, E2Fs, Rb, CKI, Cks, cyclin D, cdc25, Wee1, Nim1, MAP kinases, etc. Preferably, a cell cycle control gene will exert such regulatory control at the post-translation level, via interactions involving the polypeptide product expressed therefrom.

More specifically, cell cycle control genes are all genes involved in the control of entry and progression through S phase. They include, not exclusively, genes expressing "cell cycle control proteins" such as cyclin dependent kinases (CDK), cycline dependent kinase inhibitors (CKI), D, E and a cyclins, E2F and DP transcription factors, pocket proteins, CDC7/DBF4 kinase, CDC6, MCM2-7, Orc proteins, cdc45, components of SCF ubiquitin ligase, PCNA, and DNA-polymerase, amongst others.

The term " cell cycle control protein" includes cyclins a, B, C, D and E, including

CYCA1;1, CYCA2;1, CYCA3;1, CYCB1;1, CYCB1;2, CYCB2;2, CYCD1;1, CYCD2;1. CYCD3;1, and CYCD4;1 (Evans *et al.* 1983; Francis *et al.* 1998; Labbe *et al.* 1989; Murray and Kirschner 1989; Renaudin *et al* 1996; Soni *et al* 1995; Sorrell *et al* 1999; Swenson *et al* 1986); cyclin dependent kinase inhibitor (CKI) proteins such as ICK1 (Wang *et al* 1997), FL39, FL66, FL67 (PCT/EP98/05895), Sic1, Far1, Rum1, p21, p27, p57, p16, p15, p18, p19 (Elledge 1996; Pines 1995), p14 and p14ARF; p13suc1 or CKS1At (De Veylder *et al* 1997; Hayles and Nurse 1986) and nim-1 (Russell and Nurse 1987a; Russell and Nurse 1987b; Fantes 1989; Russell and Nurse 1986; Russell and Nurse 1987a; Russell and Nurse 1987b) homologues of Cdc2 such as Cdc2MsB (Hirt *et al* 1993) CdcMs kinase (Bögre *et al* 1997) cdc2 T14Y15 phosphatases such as Cdc25 protein phosphatase or p80cdc25 (Bell *et al* 1993; Elledge 1996; Kumaghi and Dunphy 1991; Russell and Nurse 1986) and Pyp3 (Elledge 1996) cdc2 protein kinase or p34cdc2 (Colasanti *et al* 1991; Feiler and Jacobs 1990; Hirt *et al* 1991; John *et al* 1989; Lee and Nurse 1987; Nurse and Bissett 1981; Ormond and Francis 1993) cdc2a protein kinase (Hemerly *et al* 1993) cdc2 T14Y15 kinases such as wee1 or p107wee1 (Elledge 1996; Russell and Nurse 1986; Russell and Nurse 1987a; Russell and Nurse 1987a; Sun *et al* 1999) mik1 (Lundgren *et al* 1991) and myt1 (Elledge 1996); cdc2 T161 kinases such as Cak and Civ (Elledge 1996); cdc2 T161 phosphatases such as Kap1 (Elledge 1996); cdc28 protein kinase or p34cdc28 (Nasmyth 1993; Reed *et al.* 1985) p40MO15 (Fesquet *et al* 1993; Poon *et al.* 1993) chk1 kinase (Zeng *et al* 1998) cds1 kinase (Zeng *et al* 1998) growth associated H1 kinase (Labbe *et al* 1989; Lake and Salzman 1972; Langan 1978; Zeng *et al* 1998) MAP kinases described by (Binarova *et al* 1998; Bögre *et al* 1999; Calderini *et al* 1998; Wilson *et al* 1999).

Other cell cycle control proteins are involved in cyclin D-mediated entry of cells into G1 from G0 include pRb (Xie *et al.*, 1996; Huntley *et al.*, 1998), E2F, RIP, MCM7, and the pRb-like proteins p107 and p130.

Other cell cycle control proteins are involved in the formation of a pre-replicative complex at one or more origins of replication, such as, but not limited to, ORC, CDC6, CDC14, RPA and MCM proteins or in the regulation of formation of this pre-replicative

complex, such as, but not limited to, the CDC7, DBF4 and MBF proteins.

For the present purpose, the term "cell cycle control protein" shall further be taken to include any one or more of those proteins that are involved in the turnover of any other cell cycle control protein, or in regulating the half-life of said other cell cycle control protein. The term "protein turnover" is to include all biochemical modifications of a protein leading to the physical or functional removal of said protein. Although not limited to these, examples of such modifications are phosphorylation, ubiquitination and proteolysis. Particularly preferred proteins which are involved in the proteolysis of one or more of any other of the above-mentioned cell cycle control proteins include the yeast-derived and animal-derived proteins, Skp1, Skp2, Rub1, Cdc20, cullins, CDC23, CDC27, CDC16, and plant-derived homologues thereof (Cohen-Fix and Koshland 1997; Hochstrasser 1998; Krek 1998; Lisztwan *et al* 1998) and Plesse *et al* in (Francis *et al* 1998)).

For the present purpose, the term "cell cycle control genes" shall further be taken to include any one or more of those gene that are involved in the transcriptional regulation of cell cycle control gene expression such as transcription factors and upstream signal proteins. Additional cell cycle control genes are not excluded.

For the present purpose, the term "cell cycle control genes" shall further be taken to include any cell cycle control gene or mutant thereof, which is affected by environmental signals such as for instance stress, nutrients, pathogens, or by intrinsic signals such as an animal mitogen or plant hormone (auxin, cytokinin, ethylene, gibberellic acid, abscisic acid and brassinosteroid).

In a preferred embodiment, the cell cycle control protein of the present invention is involved in controlling or regulating the length of the G2 phase of the cell cycle and/or the transition from the G2 phase to the M phase. As will be apparent from the description contained herein, a cell cycle control protein that is capable of regulating the length of the G2 phase and/or the G2/M transition will be capable of modifying the duration of the cell cycle and the time taken by a cell to exit the cell cycle and

commence cell division.

Even more preferably, the cell cycle control protein of the present invention, when expressed or over-expressed in a plant cell, tissue or organ, is capable of shortening the length of the G2 phase of the cell cycle and/or reducing the transition from the G2 phase to the M phase and, as a consequence, can be used to advance cell division.

Still more preferably, the cell cycle control protein is a cyclin protein or a homologue, analogue or derivative thereof, and still more preferably a mitogenic cyclin protein, such as, for example, a cyclin B protein or a cyclin B-like protein.

In a particularly preferred embodiment of the invention, the cell cycle control protein is a cyclin B protein comprising the alfalfa CycMs2 protein or a biologically-active homologue, analogue or derivative thereof and, in particular, a plant-derived homologue of the alfalfa CycMs2 protein. The present invention clearly contemplates the use of functional homologues of the alfalfa CycMs2 protein and is not to be limited in application to the use of nucleotide sequences encoding the alfalfa CycMs2 protein.

"Homologues" of a cyclin protein, such as cyclin B, in particular homologues of CycMs2, are those peptides, oligopeptides, polypeptides, proteins and enzymes which contain amino acid substitutions, deletions and/or additions relative to the cyclin polypeptide with respect to which they are a homologue, without altering one or more of its cell cycle control properties, in particular without reducing the cyclin B or cyclin B-like activity of the resulting polypeptide with respect to its ability to induce one or more cytokinin-mediated and/or gibberellin-mediated effects in a plant cell, tissue, organ or whole organism. For example, a homologue of the alfalfa CycMs2 polypeptide will consist of a bioactive amino acid sequence variant of said polypeptide.

To produce such homologues, amino acids present in the cyclin polypeptide can be replaced by other amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, antigenicity, propensity to form or break α-helical structures or β-sheet structures, and so on.

Substitutional variants are those in which at least one residue in the cyclin protein amino acid sequence has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1-10 amino acid residues. and deletions will range from about 1-20 residues. Preferably, amino acid substitutions will comprise conservative amino acid substitutions, such as those described *supra*.

Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the cyclin protein. Insertions can comprise amino– terminal and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid sequence will be smaller than amino or carboxyl terminal fusions, of the order of about 1 to 4 residues.

Deletional variants are characterised by the removal of one or more amino acids from the amino acid sequence of the cyclin protein.

Amino acid variants of the cyclin protein may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis and the like, or by recombinant DNA manipulations. The manipulation of DNA sequences to produce variant proteins which manifest as substitutional, insertional or deletional variants are well known in the art. For example, techniques for making substitution mutations at predetermined sites in DNA having known sequence are well known to those skilled in the art, such as by M13 mutagenesis or other site-directed mutagenesis protocol.

"Analogues" of a cyclin protein, such as cyclin B, in particular analogues of CycMs2, are defined as those peptides, oligopeptides, polypeptides, proteins and enzymes which are functionally equivalent to the cyclin with respect to which they are analogous. Analogues of a cyclin protein will preferably exhibit like bioactivity in inducing one or more cytokinin-mediated and/or gibberellin-mediated effects in plant cells, tissues,

organs or whole organisms,

Preferably, an analogue of a cyclin protein is a cell cycle control protein, or a modified cell cycle control protein, which produces the same modifications to cell fate, plant morphology, biochemistry or physiology when ectopically-expressed in a plant, as observed for the CycMs2 protein. More preferably, an analogue of a cyclin protein, in particular cyclin B, is a cell cycle control protein other than a Cdc25 protein, or a Cdc2 protein.

"Derivatives" of a cyclin protein, such as cyclin B, in particular derivatives of CycMs2, are those peptides, oligopeptides, polypeptides, proteins and enzymes which comprise at least about five contiguous amino acid residues of a naturally-occurring cyclin polypeptide, in particular the alfalfa CycMs2 polypeptide, but which retain activity in the induction of one or more cytokinin-mediated and/or gibberellin-mediated effects in a plant cell, tissue, organ or whole organism. A "derivative" may further comprise additional naturally-occurring, altered glycosylated, acylated or non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally-occurring cyclin polypeptide. Alternatively or in addition, a derivative may comprise one or more non-amino acid substituents compared to the amino acid sequence of a naturally-occurring cyclin polypeptide, for example a reporter molecule or other ligand, covalently or non-covalently bound to the amino acid sequence such as, for example, a reporter molecule which is bound thereto to facilitate its detection.

Other examples of recombinant or synthetic mutants and derivatives of a cyclin protein, such as, for example, a cyclin B protein, and, in particular the alfalfa CycMs2 polypeptide, include those molecules incorporating single or multiple substitutions, deletions and/or additions therein, such as carbohydrates, lipids and/or proteins or polypeptides. Naturally-occurring or altered glycosylated or acylated forms of cyclin B are also contemplated by the present invention. Additionally, homopolymers or heteropolymers comprising one or more copies of the cyclin polypeptide are within the scope of the invention, the only requirement being that such molecules possess biological activity in inducing one or more cytokinin-mediated and/or gibberellin-

mediated effects in plant cells, tissues, organs or whole organisms.

Particularly preferred homologues, analogues and derivatives of a cyclin protein which are contemplated for use in performing the present invention are derived from plants or capable of being expressed therein.

To effect expression of the cyclin protein in a plant cell, tissue or organ, either the protein may be introduced directly to said cell, such as by microinjection means or alternatively, an isolated nucleic acid molecule encoding said protein may be introduced into the cell, tissue or organ in an expressible format.

By "expressible format" is meant that the isolated nucleic acid molecule is in a form suitable for being transcribed into mRNA and/or translated to produce a protein, either constitutively or following induction by an intracellular or extracellular signal, such as an environmental stimulus or stress (anoxia, hypoxia, temperature, salt, light, dehydration, etc) or a chemical compound such as an antibiotic (tetracycline, ampicillin, rifampicin, kanamycin) hormone (eg. gibberellin, auxin, cytokinin, glucocorticoid, etc), hormone analogue (iodoacetic acid (IAA), 2,4-D, etc) , metal (zinc, copper, iron, etc), or dexamethasone, amongst others. As will be known to those skilled in the art, expression of a functional protein may also require one or more post-translational modifications, such as glycosylation, phosphorylation, dephosphorylation, or one or more protein-protein interactions, amongst others. All such processes are included within the scope of the term "expressible format".

Preferably, expression of a cyclin protein in a specific plant cell, tissue, or organ is effected by introducing and expressing an isolated nucleic acid molecule encoding said protein, such as a cDNA molecule, genomic gene, synthetic oligonucleotide molecule, mRNA molecule or open reading frame, to said cell, tissue or organ, wherein said nucleic acid molecule is placed operably in connection with a suitable plant-expressible promoter sequence.

Reference herein to a "promoter" is to be taken in its broadest context and includes the

transcriptional regulatory sequences derived from a classical eukaryotic genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner.

The term "promoter" also includes the transcriptional regulatory sequences of a classical prokaryotic gene, in which case it may include a -35 box sequence and/or a -10 box transcriptional regulatory sequences.

The term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of a nucleic acid molecule in a cell, tissue or organ.

Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression and/or to alter the spatial expression and/or temporal expression of a nucleic acid molecule to which it is operably connected. For example, copper-responsive, glucocorticoid-responsive, dexamethasone-responsive or tetracycline-responsive regulatory elements may be placed adjacent to a heterologous promoter sequence driving expression of a nucleic acid molecule to confer copper inducible, glucocorticoid-inducible, dexamethasone-inducible, or tetracycline-inducible expression respectively, on said nucleic acid molecule.

In the context of the present invention, the promoter is a plant-expressible promoter sequence. By "plant-expressible" is meant that the promoter sequence, including any additional regulatory elements added thereto or contained therein, is at least capable of inducing, conferring, activating or enhancing expression in a plant cell, preferably a monocotyledonous or dicotyledonous plant cell and in particular a dicotyledonous plant cell, tissue, or organ. Accordingly, it is within the scope of the invention to include any promoter sequences that also function in non-plant cells, such as yeast cells, animal cells and the like.

The terms "plant-operable" and "operable in a plant" when used herein, in respect of a promoter sequence, shall be taken to be equivalent to a plant-expressible promoter sequence.

In the present context, a "regulatable promoter sequence" is a promoter that is capable of conferring expression on a structural gene in a particular cell, tissue, or organ or group of cells, tissues or organs of a plant, optionally under specific conditions, however does generally not confer expression throughout the plant under all conditions. Accordingly, a regulatable promoter sequence may be a promoter sequence that confers expression on a gene to which it is operably connected in a particular location within the plant or alternatively, throughout the plant under a specific set of conditions, such as following induction of gene expression by a chemical compound or other elicitor.

Preferably, the regulatable promoter used in the performance of the present invention confers expression in a specific location within the plant, either constitutively or following induction, however not in the whole plant under any circumstances. Included within the scope of such promoters are cell-specific promoter sequences, tissue-specific promoter sequences, inducible promoter sequences, organ-specific promoter sequences, cell cycle specific gene promoter sequences, and constitutive promoter sequences that have been modified to confer expression in a particular part of the plant at any one time, such as by integration of said constitutive promoter within an excisable genetic element.

The term "cell-specific" shall be taken to indicate that expression is predominantly in a particular plant cell or plant cell-type, albeit not necessarily exclusively in that plant cell or plant cell-type.

Similarly, the term "tissue-specific" shall be taken to indicate that expression is predominantly in a particular plant tissue or plant tissue-type, albeit not necessarily exclusively in that plant tissue or plant tissue-type.

Similarly, the term "organ-specific" shall be taken to indicate that expression is predominantly in a particular plant organ albeit not necessarily exclusively in that plant organ.

Those skilled in the art will be aware that an "inducible promoter" is a promoter the transcriptional activity of which is increased or induced in response to a developmental, chemical, environmental, or physical stimulus.

Similarly, the term "cell cycle specific" shall be taken to indicate that expression is predominantly cyclic and occurring in one or more, not necessarily consecutive phases of the cell cycle albeit not necessarily exclusively in cycling cells.

As will be apparent from the preceding description, the present invention does not require the exclusive expression of the cyclin protein in a cell, tissue or organ of a plant, in order to induce non-pleiotropic cytokinin-mediated and/or gibberellin-mediated effects therein, subject to the proviso that expression is at least predominantly localised in a particular cell, tissue or organ of the plant. Preferably, the promoter selected for regulating expression of the cyclin protein in the plant cell, tissue or organ will confer expression in a range of cell-types or tissue-types or organs, sufficient to produce the desired phenotype, whilst avoiding undesirable phenotypes produced in other cell-types or tissue-types or organs.

More preferably, the promoter selected for regulating expression of the cyclin protein in the plant cell, tissue or organ, will confer expression in a limited number of cells or cell-types or tissues or tissue-types or organs of the plant.

Even more preferably, the promoter selected for regulating expression of the cyclin protein in the plant cell, tissue or organ, will confer expression in a single cell-type or tissue-type or organ of the plant.

Those skilled in the art will readily be capable of selecting appropriate promoter sequences for use in regulating appropriate expression of the cyclin protein from

publicly-available or readily-available sources, without undue experimentation.

In this regard, constitutive promoters can be made tissue-specific, cell-specific, cell-cycle specific or organ-specific, by adding regulatory elements from regulated tissue-specific, cell-specific, cell-cycle specific or organ-specific promoters to the constitutive promoter sequence. Alternatively, the otherwise constitutive expression activity of the promoter sequence may be regulated by integrating the promoter sequence and cell cycle control gene in one or more excisable genetic elements.

As used herein, the term "an excisable genetic element" shall be taken to refer to any nucleic acid which comprises a nucleotide sequence which is capable of integrating into the nuclear, mitochondrial, or plastid genome of a plant, and subsequently being autonomously mobilised, or induced to mobilise, such that it is excised from the original integration site in said genome. By "autonomously mobilised" is meant that the genetic element is excised from the host genome randomly, or without the application of an external stimulus to excise. In performing the present invention, the genetic element is preferably induced to mobilise, such as, for example, by the expression of a recombinase protein in the cell which contacts the integration site of the genetic element and facilitates a recombination event therein, excising the genetic element completely, or alternatively, leaving a "footprint", generally of about 20 nucleotides in length or greater , at the original integration site.

Preferably, the excisable genetic element comprises a transposable genetic element, such as, for example, *Ac, Ds, Spm,* or *En*, or alternatively, on or more loci for interaction with a site-specific recombinase protein, such as, for example, one or more *lox* or *frt* nucleotide sequences.

Known site-specific recombination systems, for example the cre/*lox* system and the flp/*frt* system which comprise a loci for DNA recombination flanking a selected gene, specifically *lox* or *frt* genetic sequences, combination with a recombinase, cre or flp, which specifically contacts said loci, producing site-specific recombination and deletion

of the selected gene. In particular, European Patent No. 0228009 (E.I. Du Pont de Nemours and Company) published 29 April, 1987 discloses a method for producing site-specific recombination of DNA in yeast utilising the cre/*lox* system, wherein yeast is transformed with a first DNA sequence comprising a regulatory nucleotide sequence and a *cre* gene and a second DNA sequence comprising a pre-selected DNA segment flanked by two *lox* sites such that, upon activation of the regulatory nucleotide sequence, expression of the *cre* gene is effected thereby producing site-specific recombination of DNA and deletion of the pre-selected DNA segment. United States Patent No. 4,959,317 (E.I. Du Pont de Nemours and Company) filed 29 April 1987 and International Patent Application No. PCT/US90/07295 (E.I. Du Pont de Nemours and Company) filed 19 December, 1990 also disclose the use of the cre/*lox* system in eukaryotic cells.

A requirement for the operation of site-specific recombination systems is that the loci for DNA recombination and the recombinase enzyme contact each other *in vivo*, which means that they must both be present in the same cell. The prior art means for excising unwanted transgenes from genetically-transformed cells all involve either multiple transformation events or sexual crossing to produce a single cell comprising *both* the loci for DNA recombination and the site-specific recombinase.

A "site-specific recombinase" is understood by those skilled in the relevant art to mean an enzyme or polypeptide molecule which is capable of binding to a specific nucleotide sequence, in a nucleic acid molecule preferably a DNA sequence, hereinafter referred to as a "recombination locus" and induce a cross-over event in the nucleic acid molecule in the vicinity of said recombination locus. Preferably, a site-specific recombinase will induce excision of intervening DNA located between two such recombination loci.

The terms "recombination locus" and "recombination loci" shall be taken to refer to any sequence of nucleotides which is recognized and/or bound by a site-specific recombinase as hereinbefore defined.

A number of different site specific recombinase systems can be used, including but not limited to the Cre/lox system of bacteriophage P1, the FLP/FRT system of yeast, the Gin recombinase of phase Mu, the Pin recombinase of *E.coli*, the PinB, PinD and PinF from *Shigella*, and the R/RS system of the psR1 plasmid. Some of these systems have already been used with high efficiency in plants, such as tobacco, and *A. thaliana.*

Preferred site-specific recombinase systems contemplated for use in the gene constructs of the invention, and in conjunction with the inventive method, are the bacteriophage P1 Cre/lox system, and the yeast FLP/FRT system. The site specific recombination loci for each of these two systems are relatively short, only 34 bp for the *lox* loci, and 47 bp for the *frt* loci.

In a most particularly preferred embodiment, however, the recombination loci are *lox* sites, such as *lox P, lox B, Lox L or lox R* or functionally-equivalent homologues, analogues or derivatives thereof. *Lox* sites may be isolated from bacteriophage or bacteria by methods known in the art (Hoess *et al.*, 1982). It will also be known to those skilled in the relevant art that *lox* sites may be produced by synthetic means, optionally comprising one or more nucleotide substitutions, deletions or additions thereto.

The relative orientation of two recombination loci in a nucleic acid molecule or gene construct may influence whether the intervening genetic sequences are deleted or excised or, alternatively, inverted when a site-specific recombinase acts thereupon. In a particularly preferred embodiment of the present invention, the recombination loci are oriented in a configuration relative to each other such as to promote the deletion or excision of intervening genetic sequences by the action of a site-specific recombinase upon, or in the vicinity of said recombination loci.

The present invention clearly encompasses the use of gene constructs which facilitate the expression of a site-specific recombinase protein which is capable of specifically contacting the excisable genetic element, in conjunction with the gene constructs

containing the cell cycle control protein-encoding gene. A single gene construct may be used to express both the site-specific recombinase protein and the cell cycle control protein, or alternatively, these may be introduced to plant cells on separate gene constructs.

For example, the recombinase gene could already be present in the plant genome prior to transformation with the gene construct of the invention, or alternatively, it may be introduced to the cell subsequent to transformation with the gene construct of the invention, such as, for example, by a separate transformation event, or by standard plant breeding involving hybridisation or cross-pollination. In one embodiment of the current invention, the recombinase gene is supplied to the transgenic plants containing a vector backbone sequence flanked by recombination sites by sexual crossing with a plant containing the recombinase gene in it's genome. Said recombinase can be operably linked to either a constitutive or an inducible promoter. The recombinase gene can alternatively be under the control of single subunit bacteriophage RNA polymerase specific promoters, such as a T7 or a T3 specific promoter, provided that the host cells also comprise the corresponding RNA polymerase in an active form. Yet another alternative method for expression of the recombinase consists of operably linking the recombinase open reading frame with an upstream activating sequence fired by a transactivating transcription factor such as GAL4 or derivatives (US5801027, WO97/30164, WO98/59062) or the Lac repressor (EP0823480), provided that the host cell is supplied in an appropriate way with the transcription factor.

Alternatively, a substantially purified recombinase protein could be introduced directly into the eukaryotic cell, eg., by micro-injection or particle bombardment. Typically, the site-specific recombinase coding region will be operably linked to regulatory sequences enabling expression of the site-specific recombinase in the eukaryotic cell. In a preferred embodiment of the present invention, the site-specific recombinase sequences is operably linked to an inducible promoter.

Dual-specific recombinase systems can also be employed, which may employ a

recombinase enzyme in conjunction with direct or indirect repeats of two different site-specific recombination loci corresponding to the dual-specific recombinase, such as that described in International Patent Publication No. WO99/25840.

As will be known to those skilled in the art, for recombination mediated by a transposon to occur, a pair of DNA sequences comprising inverted repeat transposon border sequences, flanking the excisable genetic element sequence, and a specific transposase enzyme, are required. The transposase catalyzes a recombination reaction only between two transposon border sequences.

A number of different plant-operable transposon/transposase systems can be used including but not limited to the *Ac/Ds* system, the *Spm* system and the *Mu* system. All of these systems are operable in *Zea mays*, and at least the *Ac/Ds* and the *Spm* system function in other plants.

Preferred transposon sequences for use in the gene constructs of the invention are the *Ds*-type and the *Spm*-type transposons, which are delineated by border sequences of only 11 bp and 13 bp in length, respectively.

As with the use of site-specific recombinase systems, the present invention clearly encompasses the use of gene constructs which facilitate the expression of a transposase enzyme which is capable of specifically contacting the transposon border sequence, in conjunction with the gene constructs containing thecell cycle control protein-encoding gene. A single gene construct may be used to express both the transposase and the cell cycle control protein, or alternatively, these may be introduced to plant cells on separate gene constructs.

For example, the transposase-encoding gene could already be present in the plant genome prior to transformation with the gene construct of the invention, or alternatively, it may be introduced to the cell subsequent to transformation with the gene construct of the invention, such as, for example, by a separate transformation

event, or by standard plant breeding involving hybridisation or cross-pollination. Alternatively, a substantially purified transposase protein could be introduced directly into the eukaryotic cell, eg., by micro-injection or particle bombardment. Typically, the transposase coding region will be operably linked to regulatory sequences enabling expression of the transposase in the eukaryotic cell. In a preferred embodiment of the present invention, the transposase-encoding sequence is operably linked to an inducible promoter.

In the present context, transposon border sequences are organized as inverted repeats flanking the excisable genetic element. As transposons often re-integrate at another locus of the host's genome, segregation of the progeny of the hosts in which the transposase was allowed to act might be necessary to separate transformed hosts containing only the gene(s) of interest and transformed hosts containing only the cell cycle control protein-encoding gene.

Likewise, the site-specific recombinase gene or transposase gene present in the host's genome can be removed by segregation of the progeny of the hosts to separate transformed hosts containing only the gene(s) of interest and transformed hosts containing only the site-specific recombinase gene or transposase gene. Alternatively, said site-specific recombinase gene or transposase gene are included in the same or in a different excisable genetic element as thecell cycle control protein-encoding gene.

Placing a nucleic acid molecule under the regulatory control of a promoter sequence, or in operable connection with a promoter sequence, means positioning said nucleic acid molecule such that expression is controlled by the promoter sequence.

A promoter is usually, but not necessarily, positioned upstream, or at the 5′-end, and within 2 kb of the start site of transcription, of the nucleic acid molecule which it regulates.

In the construction of heterologous promoter/structural gene combinations it is

generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting (i.e., the gene from which the promoter is derived). As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting (i.e., the gene from which it is derived). Again, as is known in the art, some variation in this distance can also occur.

Examples of promoters suitable for use in gene constructs of the present invention include those listed in Table 1, amongst others. The promoters listed in Table 1 are provided for the purposes of exemplification only and the present invention is not to be limited by the list provided therein. Those skilled in the art will readily be in a position to provide additional promoters that are useful in performing the present invention.

In an alternative embodiment, the promoter is a tissue-specific inducible promoter sequence, such as but not limited to a light-inducible *rbcs-1A* or *rbcs-3A* promoter, anoxia-inducible maize *Adh1* gene promoter (Howard *et al.*, 1987; Walker *et al.*, 1987), hypoxia-inducible maize *Adh1* gene promoter (Howard *et al.*, 1987; Walker *et al.*, 1987), and the temperature-inducible heat shock promoter. Such environmentally-inducible promoters are reviewed in detail by Kuhlemeier *et al.* 1987).

In an alternative embodiment, the promoter is a chemically-inducible promoter, such as the 3-β- indoylacrylic acid-inducible *Tip* promoter; IPTG-inducible *lac* promoter; phosphate-inducible promoter; L-arabinose-inducible *araB* promoter; heavy metal-inducible metallothionine gene promoter; dexamethasone-inducible promoter; glucocorticoid-inducible promoter; ethanol-inducible promoter (Zeneca); the N,N-diallyl-2,2-dichloroacetamide-inducible glutathione-S-transferase gene promoter (Wiegand *et al.*, 1986); or any one or more of the chemically-inducible promoters described by Gatz *et al.* (1996), amongst others.

In an alternative embodiment, the promoter is a wound-inducible or pathogen-inducible promoter, such as the phenylalanine ammonia lyase (PAL) gene promoter (Ebel *et al.*, 1984), chalcone synthase gene promoter (Ebel *et al.*, 1984) or the potato wound-inducible promoter (Cleveland *et al.*, 1987), amongst others.

In a further alternative embodiment, the promoter is a hormone-inducible promoter, such as the abscisic acid-inducible wheat 7S globulin gene promoter and the wheat Em gene promoter (Marcotte *et al.*,1988);an auxin-responsive gene promoter, such as, for example, the *SAUR* gene promoter, the *parAs* and *parAt* gene promoters(van der Zaal *et al.*, 1991; Gil *et al.*, 1994; Niwa *et al.*, 1994); or a gibberellin-inducible promoter such as the *Amy32b* gene promoter (Lanahan *et al.* 1992), amongst others.

In a further alternative embodiment, the promoter is a constitutive plant-expressible promoter sequence such as the CaMV 35S promoter sequence , CaMV 19S promoter sequence, the octopine synthase (OCS) promoter sequence , or nopaline synthase (NOS) promoter sequence (Ebert *et al.* 1987), amongst others.

In the case of constitutive promoters or promoters that induce expression throughout the entire plant, it is preferred that such sequences are modified by the addition of nucleotide sequences derived from one or more of the tissue-specific promoters listed in Table 1, or alternatively, nucleotide sequences derived from one or more of the above-mentioned tissue-specific inducible promoters, to confer tissue-specificity thereon. For example, the CaMV 35S promoter may be modified by the addition of maize *Adh1* promoter sequence, to confer anaerobically-regulated root-specific expression thereon, as described previously (Ellis *et al.*, 1987). Such modifications can be achieved by routine experimentation by those skilled in the art.

In yet another alternative embodiment, the promoter is a cell cycle specific gene promoter, such as, for example, the Cdc2a promoter sequence (Chung and Parish 1995) or the PCNA promoter sequence (Kosugi *et al*, 1991, Kosugi and Ohashi 1997).

Preferred embodiments of the invention relate to the effect(s) of cytokinins on the

determination of cell fate and plant development and cellular processes therefor. However, the present invention clearly contemplates the broad application of the inventive method to the modification of a range of cellular processes involved in determining cell fate and plant cell development, including but not limited to modifying the length of the cell cycle, and in particular, modifying the length of the G2 phase; modifying the duration of the G2/M phase transition; the advancement of cell division; determination of cell fate and in particular root development and/or seed development; the modification of source/sink relationships, and/or the inhibition of root growth and/or inhibition of root apical dominance and/or the delay of senescence and/or modifying shoot apical dominance.

Preferred embodiments of the invention also relate to specific the effect(s) of hormones such as cytokinins and/or gibberellins on plant metabolism. With respect to such hormone-related effects, the present invention clearly contemplates the broad application of the inventive method to the modification of a range of cellular processes that are mediated by cytokinins and/or gibberellins, including but not limited to cellular development and/or cell fate; the advancement of cell division;the modification of source/sink relationships, and/or the inhibition of root growth and/or the inhibition of root apical dominance and/or the delay of senescence and/or the initiation, promotion, stimulation or enhancement of seed development and/or tuber formation and/or shoot initiation and/or dwarfism and/or pigment synthesis and/or the modifcation of shoot apical dominance (i.e. bushiness) of the plant.

Accordingly, in a particularly preferred embodiment of the present invention, there is provided a method of shortening the duration of the G2 phase of the cell cycle and/or shortening the G2/M phase transition of a cell comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a regulatable promoter sequence as described *supra*.

TABLE 1

EXEMPLARY PLANT-OPERABLE PROMOTERS FOR USE IN PERFORMING THE PRESENT INVENTION

I: CELL-SPECIFIC, TISSUE-SPECIFIC, AND ORGAN-SPECIFIC PROMOTERS		
GENE SOURCE	**EXPRESSION PATTERN**	**REFERENCE**
α-amylase (Amy32b)	aleurone	Lanahan *et al.*, 1992; Skriver *et al.*, 1991
cathepsin β-like gene	aleurone	Cejudo *et al.*,1992.
Agrobacterium rhizogenes rolB	cambium	Nilsson *et al.*, 1997
PRP genes	cell wall	http://salus.medium.edu/mmg/tierney/html
AtPRP4	flowers	http://salus.medium.edu/mmg/tierney/html
chalene synthase (chsA)	flowers	Van der Meer *et al.*, 1990.
LAT52	anther	Twell *et al.*,1989
apetala-3	flowers	
chitinase	fruit (berries, grapes, etc)	Thomas *et al.* CSIRO Plant Industry, Urrbrae, South Australia, Australia; http://winetitles.com.au/gwrdc/ csh95-1.html
rbcs-3A	green tissue (eg leaf)	Lam *et al.*, 1990; Tucker *et al.*, 1992.
leaf-specific genes	leaf	Baszczynski *et al.*, 1988.
AtPRP4	leaf	http://salus.medium.edu/mmg/tierney/html
Pinus cab-6	leaf	Yamamoto *et al.*, 1994.
SAM22	senescent leaf	Crowell *et al.*, 1992.
R. japonicum nif gene	nodule	United States Patent No. 4, 803, 165
B. japonicum nifH gene	nodule	United States Patent No. 5, 008, 194
GmENOD40	nodule	Yang *et al.* 1993
PEP carboxylase (PEPC)	nodule	Pathirana *et al.*, 1992.

leghaemoglobin (Lb)	nodule	Gordon *et al.*, 1993.
Tungro bacilliform virus gene	phloem	Bhattacharyya-Pakrasi *et al.*, 1992.
sucrose-binding protein gene	plasma membrane	Grimes *et al.*, 1992.
pollen-specific genes	pollen; microspore	Albani *et al.*, 1990; Albani *et al.*, 1991
Zm13	pollen	Guerrero *et al.*, 1993
apg gene	microspore	Twell *et al.*, 1993
maize pollen-specific gene	pollen	Hamilton *et al.*, 1992.
sunflower pollen-expressed gene	pollen	Baltz *et al.*, 1992.
B. napus pollen-specific gene	pollen;anther; tapetum	Arnoldo *et al.*, 1992.
root-expressible genes	roots	Tingey *et al.*, 1987.
tobacco auxin-inducible gene	root tip	Van der Zaal *et al.*, 1991.
β-tubulin	root	Oppenheimer *et al.*, 1988.
tobacco root-specific genes	root	Conkling *et al.*, 1990.
B. napus G1-3b gene	root	United States Patent No. 5, 401, 836
SbPRP1	root	Suzuki *et al.*, 1993.
AtPRP1; AtPRP3	roots; root hairs	http://salus.medium.edu/mmg/tierney/html
RD2 gene	root cortex	http://www2.cnsu.edu/ncsu/research
TobRB7 gene	root vasculature	http://www2.cnsu.edu/ncsu/research
AtPRP4	leaves; flowers; lateral root primordia	http://salus.medium.edu/mmg/tierney/html
seed-specific genes	seed	Simon *et al.*, 1985; Scofield *et al.*, 1987; Baszczynski *et al.*, 1990.
Brazil Nut albumin	seed	Pearson *et al.*, 1992.
legumin	seed	Ellis *et al.*, 1988.
glutelin (rice)	seed	Takaiwa *et al.*, 1986; Takaiwa *et al.*, 1987.
zein	seed	Matzke *et al.*, 1990
napA	seed	Stalberg *et al.*, 1996.

wheat LMW and HMW glutenin-1	endosperm	Mol Gen Genet 216:81-90, 1989; NAR 17:461-2, 1989
wheat SPA	seed	Albani *et al.*,1997
wheat α, β, γ-gliadins	endosperm	EMBO 3:1409-15, 1984
barley ltr1 promoter	endosperm	
barley B1, C, D, hordein	endosperm	Theor Appl Gen 98:1253-62, 1999; Plant J 4:343-55, 1993; Mol Gen Genet 250:750-60, 1996
barley DOF	endosperm	Mena *et al.*, 1998
blz2	endosperm	EP99106056.7
synthetic promoter	endosperm	Vicente-Carbajosa *et al.*, 1998.
rice prolamin NRP33	endosperm	Wu *et al.*, 1998
rice α-globulin Glb-1	endosperm	Wu *et al.*, 1998
rice OSH1	embryo	Sato *et al.*,1996
rice α-globulin REB/OHP-1	endosperm	Nakase *et al.*,1997
rice ADP-glucose PP	endosperm	Trans Res 6:157-68, 1997
maize ESR gene family	endosperm	Plant J 12:235-46, 1997
sorgum γ-kafirin	endosperm	PMB 32:1029-35, 1996
KNOX	embryo	Postma-Haarsma *et al.*, 1999
rice oleosin	embryo and aleurone	Wu et at,1998
sunflower oleosin	seed (embryo and dry seed)	Cummins *et al.*, 1992
LEAFY	shoot meristem	Weigel *et al.*, 1992.
Arabidopsis thaliana knat1	shoot meristem	Accession number AJ131822
Malus domestica kn1	shoot meristem	Accession number Z71981
CLAVATA1	shoot meristem	Accession number AF049870
stigma-specific genes	stigma	Nasrallah *et al.*, 1988; Trick *et al.*, 1990
class I patatin gene	tuber	Liu *et al.*, 1991.
PCNA rice	meristem	Kosugi *et al.*, 1991; Kosugi *et al.*, 1997.

Pea TubA1 tubulin	Dividing cells	Stotz *et al.*,1999
Arabidopsis cdc2a	cycling cells	Chung and Parish, 1995
Arabidopsis Rop1A	Anthers; mature pollen; pollen tube	Li *et al.*, 1998
Arabidopsis AtDMC1	Meiosis-associated	Klimyuk and James,1997
Pea PS-IAA4/5 and PS-IAA6	Auxin-inducible	Wong *et al.*, 1996
Pea farnesyltransferase	Meristematic tissues; phloem near growing tissues; light- and sugar-repressed	Zhou *et al.*, 1997
Tobacco (N. sylvestris) cyclin B1;1	Dividing cells / meristematic tissue	Trehin *et al.*, 1997
Catharanthus roseus Mitotic cyclins CYS (A-type) and CYM (B-type)	Dividing cells / meristematic tissue	Ito *et al.*, 1997
Arabidopsis cyc1At (=cyc B1;1) and cyc3aAt (A-type)	Dividing cells / meristematic tissue	Shaul *et al.*, 1996
Arabidopsis tef1 promoter box	Dividing cells / meristematic tissue	Regad *et al.*, 1995
Catharanthus roseus cyc07	Dividing cells / meristematic tissue	Ito *et al.*, 1994

II: EXEMPLARY CONSTITUTIVE PROMOTERS

GENE SOURCE	EXPRESSION PATTERN	REFERENCE
Actin	constitutive	McElroy *et al.*, 1990
CAMV 35S	constitutive	Odell *et al.*, 1985
CaMV 19S	constitutive	Nilsson *et al.*, 1997
GOS2	constitutive	de Pater *et al.*,1992
ubiquitin	constitutive	Christensen *et al.*, 1992
rice cyclophilin	constitutive	Buchholz *et al.*, 1994
maize H3 histone	constitutive	Lepetit *et al.*, 1992
actin 2	constitutive	An *et al.*, 1996

III: EXEMPLARY STRESS-INDUCIBLE PROMOTERS

NAME	STRESS	REFERENCE
P5CS (delta(1)-pyrroline-5-carboxylate syntase)	salt, water	Zhang *et al.*, 1997
cor15a	cold	Hajela *et al.*, 1990
cor15b	cold	Wilhelm and Thomashow, 1993
cor15a (-305 to +78 nt)	cold, drought	Baker *et al.*, 1994
rd29	salt, drought, cold	Kasuga *et al.*, 1999
heat shock proteins, including artificial promoters containing the heat shock element (HSE)	heat	Barros *et al.*, 1992. Marrs *et al.*, 1993. Schoffl *et al.*, 1989.
smHSP (small heat shock proteins)	heat	Waters *et al.*, 1996
wcs120	cold	Ouellet *et al.*, 1998
ci7	cold	Kirch *et al.*, 1997
Adh	cold, drought, hypoxia	Dolferus *et al.*, 1994
pwsi18	water: salt and drought	Joshee *et al.*, 1998
ci21A	cold	Schneider *et al.*, 1997
Trg-31	drought	Chaudhary *et al.*, 1996
osmotin	osmotic	Raghothama *et al.*, 1993
lapA	wounding, enviromental	WO99/03977 University of California/INRA

IV: EXEMPLARY PATHOGEN-INDUCIBLE PROMOTERS		
NAME	PATHOGEN	REFERENCE
RB7	Root-knot nematodes (Meloidogyne spp.)	US5760386 - North Carolina State University; Opperman *et al.*, 1994
PR-1, 2, 3, 4, 5, 8, 11	fungal, viral, bacterial	Ward *et al.*, 1991; Reiss and Bryngelsson, 1996; Lebel *et al.*, 1998; Melchers *et al.*, 1994; Lawton *et al.*, 1992
HMG2	nematodes	WO9503690 - Virginia Tech Intellectual Properties Inc.
Abi3	Cyst nematodes (Heterodera spp.)	unpublished
ARM1	nematodes	Barthels *et al.*, 1997; WO 98/31822 – Plant Genetic Systems
Att0728	nematodes	Barthels *et al.*, 1997; PCT/EP98/07761
Att1712	nematodes	Barthels *et al.*, 1997; PCT/EP98/07761
Gst1	Different types of pathogens	Strittmatter *et al.*, 1996
LEMMI	nematodes	WO 92/21757 – Plant Genetic Systems
CLE	geminivirus	PCT/EP99/03445 - CINESTAV
PDF1.2	Fungal including *Alternaria brassicicola* and *Botrytis cinerea*	Manners *et al.*, 1998
Thi2.1	Fungal – *Fusarium oxysporum f sp. matthiolae*	Vignutelli *et al.*, 1998
DB#226	nematodes	Bird and Wilson, 1994; WO 95.322888
DB#280	nematodes	Bird and Wilson, 1994; WO 95.322888
Cat2	nematodes	Niebel *et al.*, 1995
αTub	nematodes	Aristizabal *et al.* (1996), 8th International Congress on Plant-Microbe Interaction, Knoxville US B-29
sHSP	nematodes	Fenoll *et al.*, 1997
Tsw12	nematodes	Fenoll *et al.*, 1997
Hs1(pro1)	nematodes	WO 98/122335 - Jung
nsLTP	viral, fungal, bacterial	Molina *et al.*, 1993
RIP	viral, fungal	Turner *et al.*, 1997

In the present context, the term "substrate of cyclin B" shall be taken to refer to any protein that interacts with cyclin B or cyclin B/CDK complex in regulating the plant cell cycle, including, but not limited to cyclin-dependent kinases (CDKs), the most significant of which is cdc2, which in all cells is the key enzyme driving entry into mitosis.

The term "modified substrate of cyclin B" refers to a homologue, analogue or derivative of a substrate of cyclin B that mimics the effect of alfalfa CycMs2 activity described herein. For example, substitution of amino acids in a cyclin-dependent kinase (CDK) can produce one or more cytokinin-like effects in a plant that are similar to those observed following constitutive cyclin B expression in the plant.

Accordingly, similar effects to the cyclin B-induced effects obtained by expressing alfalfa CycMs2 under control of the regulatable promoter, can be obtained by expressing the cyclin B substrate or a modified form thereof operably under control of the same or a functionally-equivalent promoter. The present invention clearly extends to such arrangements.

The present invention extends further to the co-expression of cyclin B, in particular alfalfa CycMs2, and one or more cyclin B substrates and/or one or more modified cyclin B substrates, operably under the control of a regulatable promoter that is selected for a particular application as described herein. The present invention also extends to the co-expression of cyclin B with another synergistic or non-antagonistic cell cycle control protein, such as Cdc25, amongst others.

In another particularly preferred embodiment of the present invention, there is provided a method of advancing cell division in a plant cell, tissue or organ comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a regulatable promoter sequence.

By "advancing cell division" is meant that the duration between the commencement of the cell cycle and entry of the cell into mitosis or alternatively or in addition, the duration between the commencement of the G2 phase of the cell cycle and entry of the cell into mitosis is shortened. In the present context this is achieved by reducing the duration of the cell in the G2 phase of the cell cycle and/or by reducing the duration of the G2/M phase transition.

In yet another particularly preferred embodiment of the present invention, there is provided a method of altering cell fate or development in a plant cell, tissue, organ or whole plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a regulatable promoter sequence.

Preferably, the cell fate or development that is altered or modified by the performance of the present invention comprises a process that is regulated by the G2 phase of the cell cycle and/or by the duration of G2 and/or by the duration of the G2/M phase transition. In a more preferred embodiment, the cell fate or development comprises root development; and/or seed development, in particular grain production and yield; and/or one or more sink/source relationships of the plant, such as carbon partitioning; and/or tissue sensecence. Other processes are not excluded.

In another particularly preferred embodiment of the present invention, there is provided a method of modifying sink/source relationships of a plant tissue, organ or whole plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a regulatable promoter sequence.

As will be known to those skilled in the art, the term "sink/source relationship" refers to the flux of carbon, in particular, in the form of sucrose or triose or triose phosphate, or other carbon-containing compound, from a source organelle, cell, tissue or organ,

such as but not necessarily limited to photosynthetic tissues (eg. flag leaves), to a particular storage organelle, cell, tissue or organ (the "sink") and to the regulation of carbon flux and/or storage in the sink by such a process. Accordingly, sink/source relationships include such processes as carbon partitioning between sucrose and starch within a particular cell, and the regulation of seed starch deposition by photosynthesis and/or photosynthate transport to the endosperm (i.e. source limitation), and by sink-limiting processes such as endosperm or storage cotyledon ATP/Pi ratio, and the level of starch-metabolising enzymes (eg. ADP-glucose pyrophosphorylase; starch synthase) within the endosperm or storage cotyledon, amongst others. Those skilled in the art will be aware that sink strength is possibly the most important yield component.

Cytokinins are known to promote phloem unloading of metabolites, and in immature seeds cell division activity is correlated with a high endogenous cytokinin level, particularly in maize and legumes. Likewise, hexoses in very young *Vicia faba* seeds stimulate cell division while sucrose stimulates starch formation at a later stage of seed development. Sucrose-to-hexose conversion is controlled by invertase and indeed invertase activity is high in very young seeds. Hexose-to-sucrose conversion is controlled by sucrose phosphate synthase, leading eventually to starch synthesis. Invertase converts sucrose in a sink (meristem, storage organ, seed, etc.) into glucose while glucose stimulates cell division. Cell division produces cell mass in the sink. Cytokinins are part of the signal transduction chain linking the incoming sucrose to the activation of cell divisions. Cytokinins act downstream of the sucrose-to-hexose conversion regulated by invertase, but may have a pleiotropic effect on invertase and the G2/M transition (and possibly also on G1/S transition). At some point in seed development, invertase activity goes down, or glucose is activately converted into starch by activation of sucrose phosphate synthase. Cell division activity is stopped, to be followed by cell expansion (including endoreduplication) and starch biosynthesis. In seeds, grain filling occurs, whilst in leaf initials on meristems, leaf expansion and development occurs. Whilst not being bound by any theory or mode of action, ectopic overexpression of a mitotic cyclin, such as CycMs2, has the same effect as cytokinin

in that it drives the cell precociously into mitosis, resulting in a shortened G2 phase. A short G2 phase (and/or a short G1 phase) are defining features of a cell that is actively-dividing. Thus, in a mitotic cyclin-overexpressing cell, the G2/M transition is uncoupled from its normal external signal, glucose and cytokinin.

In one application of this phenomenon, such an uncoupling of sink/source relationships is useful in regulating the production of seeds. For example, a transgenically-controlled mitotic cyclin level in young seeds can render these cells more or less responsive to the incoming glucose/cytokinin signal, resulting in more or fewer cells in the endosperm of the seed until glucose-to-sucrose conversion occurs.

In a further application, the production of potato tubers may be regulated. In potato tubers, sucrose induces stolon tips to develop into tubers. However, cytokinin is required to initiate cell divisions in the stolon. A role for glucose in activation of cell division is not known. Ectopic expression of mitotic cyclins, such as, for example, a cyclin B protein and, in particular, the alfalfa CycMs2 protein, can substitute for the cytokinin effect and stimulate cell division independently from the incoming tuberization signal.

Accordingly, it is possible to increase or decrease sink strength of the seed or tuber, and, by extrapolation, of any other sink. Being the work horses of the cell cycle and the endpoints of the signal transduction chain originating from the incoming sucrose. CDKs might be the final and crucial determinants of sink strength.

The promoter selected for use according to this embodiment may be any promoter sequence operable in the tissue or tissues in which carbon flux is to be modified.

Accordingly, in a related embodiment of the present invention, there is provided a method of increasing seed set and/or seed size and/or seed production and/or grain

yield in a plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B, and in particular that mimics the effect of alfalfa CycMs2, operably under the control of a seed-specific promoter sequence.

Preferably, the seed-specific promoter is operable in the seeds of monocotyledonous plants, for example the barley *Amy32b* gene promoter, Cathepsin β-like gene promoter, wheat ADP-glucose pyrophosphorylase gene promoter, maize zein gene promoter, or rice glutelin gene promoter. In an alternative embodiment, the seed-specific promoter is operable in the seeds of dicotyledonous plant species, for example the legumin gene promoter, *napA* gene promoter, Brazil Nut albumin gene promoter, pea vicilin gene promoter and sunflower oleosin gene promoter, amongst others.

Those skilled in the art will be aware that grain yield in crop plants is largely a function of the amount of starch produced in the endosperm of the seed. The amount of protein produced in the endosperm is also a contributing factor to grain yield. In contrast, the embryo and aleurone layers contribute little in terms of the total weight of the mature grain. By virtue of being linked to cell expansion and metabolic activity, endoreplication and endoreduplication are generally considered as an important factor for increasing yield (Traas *et al* 1998). As grain endosperm development initially includes extensive endoreplication (Olsen *et al* 1999), enhancing, promoting or stimulating this process is likely to result in increased grain yield. Enhancing, promoting or stimulating cell division during seed development is an alternative way to increase grain yield. As shown herein, cyclin B cooperates with Cdc25 to override the DNA synthesis checkpoint in cells. This can produce endoreplication and endoreduplication, and stimulate cell division.

Accordingly, in a preferred embodiment, the cyclin protein-encoding gene, preferably the *CycMs2* gene, is placed operably in connection with a promoter that is operable in the endosperm of the seed, in which case the combination of the cell cycle-control

protein and endosperm-expressible promoter provides the additional advantage of increasing the grain size and grain yield of the plant.

Endosperm-specific promoters that can be used to drive cyclin protein expression have been identified. The components of the promoters responsible for specific expression have been identified (Grosset et al (1997) and are interchangeable between agriculturally important cereals (Olsen et al 1992; Russell and Fromm, 1997). Several promoters can be used, including the barley *blz2* gene promoter, the rice prolamin NRP33 promoter, the rice REB promoter, the zein (ZmZ27) gene promoter, the rice glutelin 1 gene (osGT1) promoter, the rice small subunit ADP-glucose pyrophosphorylase (osAGP) promoter, the maize granule-bound starch synthase (Waxy) gene (zmGBS) promoter surveyed by Russell and Fromm (1997), the Brazil Nut albumin gene promoter, and the pea vicilin gene promoter, amongst others. Promoters derived from those genes that are expressed in the endosperm during nuclear proliferation are also useful for driving cyclin protein expression. Promoters derived from those genes that are expressed in the endosperm at the stage when nuclear proliferation is ending could be ideal for extending this period.

A three way correlation exists between cytokinin level in the endosperm, the number of endosperm cells formed during seed development and grain size, in which cytokinin activates Cdc25 enzyme which in turn activates Cdc2 kinase to drive nuclear division. Accordingly, ectopic expression of the cyclin-encoding gene in the endosperm enhances Cdc2 activation and nuclear proliferation, resulting in increased grain size, without incurring the non-specific side effects that application of cytokinin or expression of the *ipt* gene would produce in the plant.

A further advantage of the present inventive approach is that the activity of cytokinin metabolising enzymes is circumvented by the direct raising of cyclin protein activity in the endosperm, by the ectopic expression of the cyclin-encoding gene, in particular the *CycMs2* gene, therein. In cases where exogenous cytokinin is used to increase grain

size and/or endosperm size, the elevated cytokinin levels and nuclear division in the grain are curtailed by an increase in the activities of cytokinin degrading enzymes, including cytokinin oxidase (Chatfield and Armstrong 1987; reviewed by Morris *et al* 1993).

In another related embodiment of the present invention, there is provided a method of increasing tuber formation and/or development in a tuberous crop plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B, in particular that mimics the effect of alfalfa CycMs2,operably under the control of a tuber-specific promoter sequence.

Preferably, the tuberous crop plant is potato and the tuber-specific promoter is the potato patatin gene promoter. Additional species and promoters are not excluded.

In another particularly preferred embodiment of the present invention, there is provided a method of inhibiting root development and/or root growth from plant cell, meristem or other tissue, organ or whole plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a regulatable promoter sequence.

By inhibiting "root development" is meant that the formation of a root structure or root-like structure from a meristem is prevented or delayed or repressed, irrespective of whether or not the meristem is developmentally committed to forming a root structure (i.e. irrespective of whether or not the meristem is a root meristem).

By inhibiting "root growth" is meant that the continued growth of a committed root structure or root-like structure from an existing root or root meristem is prevented,

delayed, or represseds.

Accordingly, this embodiment of the present invention relates to the prevention of visible root structures or root-like structures from appearing in plants. As exemplified herein, the present inventors have demonstrated that the ectoptic expression of alfalfa CycMs2 in transgenic tobacco plants inhibits the auxin-mediated development of roots from cultured leaf disc tissue. Accordingly, the ectopic expression of cyclin B proteins in plant cells is capable of antagonising auxin-mediated processes in plants, compatible with the concept that to initiate such processes, in particular root development, the cell must remain in the G2 phase of the cell cycle for a longer period than would otherwise be the case. Whilst not being bound by any theory or mode of action, since ectopic expression of CycMs2 shortens G2 and/or the G2/M phase transition, cells do not remain in G2 for a sufficient time to initiate auxin-mediated processes such as root development.

In the performance of this embodiment of the invention, it is preferred that the promoter selected for regulating cyclin B expression is a root-expressible or meristem-expressible promoter sequence such as those listed in Table 1 and in particular, the meristem-expressible PCNA promoter sequence. However, since this embodiment of the invention is also applicable to the prevention of root regeneration in cultured cells and tissues, it will also be apparent that any promoter that is operable in the cell or tissue where inhibition of root regeneration is desired will be useful. For example, a leaf-operable promoter is preferred for use in preventing root regeneration from leaf disc tissue. Alternatively, any regulatable constitutive promoter may also be useful in performing this embodiment of the invention.

In another particularly preferred embodiment of the present invention, there is provided a method of delaying senescence of a plant tissue, organ or whole plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B operably under the

control of a regulatable promoter sequence. This embodiment of the present invention also relates to the prevention, delay or reduction of leaf chlorosis and/or leaf necrosis in plants.

Preferably, the promoter selected for use in performing this embodiment of the invention is operable in the green tissues of the plant and in particular, in the leaves. Accordingly, the use of a strong promoter such as one of the known *Cab* promoters, the SAM22 promoter, or the *rbcs-1A* and *rbcs-3A* gene promoters listed in Table 1. The SAM22 gene promoter is particularly preferred in light of the developmental regulation of the SAM22 gene and its induction in senescent leaves.

In another preferred embodiment of the present invention, there is provided a method of modifying shoot apical dominance or bushiness of a plant, comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B, in particular that mimics the effect of alfalfa CycMs2, operably under the control of a meristem-specific promoter sequence or a stem-specific promoter sequence.

Without being bound by any theory or mode of action, the shortened G2 transition modifies cellular metabolism at the level of carbon partitioning to modify th degree of branch formation in the plant, thereby modfying auxin-induced apical dominance in the plant.

In another particularly preferred embodiment of the present invention, there is provided a method of increasing the strength and/or thickness and/or stability and/or wind-resistance of a plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a stem-expressible promoter sequence.

Preferably, the stem-expressible promoter sequence is derived from the *rbcs-1A* gene, the *rbcs-3A* gene, the *AtPRP4* gene, the *T. bacilliform* virus gene, or the sucrose-binding protein gene set forth in Table 1, or a stem-specific or stem-expressible homologue, analogue or derivative thereof.

In another preferred embodiment of the present invention, there is provided a method of modifying the lignin content of a woody crop plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B, and in particular that mimics the effect of alfalfa CycMs2, operably under the control of a cambium-specific or vascular-tissue-specific promoter sequence.

Preferably, the promoter is a cinnamoyl alcohol dehydrogenase (CAD) gene promoter, laccase gene promoter, cellulose synthase gene promoter and xyloglucan endotransglucosylase (XET) gene promoter sequences, amongst others. The *T. bacilliform* virus gene promoter and the sucrose-binding protein gene promoter are also useful for this application of the invention.

Preferred target plant species according to this embodiment are woody plants of economic/ agronomic value, in particular hardwood crop plants such as, but not limited to *Eucalyptus spp., Populus spp., Quercus spp., Acer spp., Juglans spp., Fagus spp., Acacia spp.,* or teak, amongst others. More preferably, this embodiment of the invention is applicable to modifying the lignin content of *Eucalyptus spp.,* in particular *E. globulus* and *E. robusta;* or *Quercus spp.,* in particular *Q. dentata, Q. ilex, Q. incana,* and *Q. robur;Acacia spp.,* in particular *A. brevispica, A. bussei, A. drepanolobium, A. nilotica, A. pravissima,* and *A. seyal; Acer spp.,* in particular *A. pseudoplatanus* and *A. saccharum*. Additional species are not excluded.

Without being bound by any theory or mode of action, the ectopic expression of cyclin B under control of a promoter that is operable in vascular tissue and preferably, in

cambial cells, will produce thick-stemmed plants and a higher ratio of vascular tissue-to-pith cells within the stem, thereby resulting in more lignin production. Within the vascular tissue, cambial cells contain the highest levels of auxins and are therefore the preferential tissue for cyclin B overproduction.

In another preferred embodiment of the present invention, there is provided a method of modifying lateral root production in a plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B, in particular that mimics the effect of alfalfa CycMs2, operably under the control of a root-specific promoter sequence.

Preferred promoter sequences according to this embodiment of the present invention include any one of the root-expressible or root-specific promoters listed in Table 1 and in particular, the tobacco auxin-inducible gene promoter described by Van der Zaal *et al* (1991) that confers expression in the root tip of plants, in particular dicotyledonous plants.

In yet another preferred embodiment of the present invention, there is provided a method of modifying the nitrogen-fixing capability of a plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B, in particular that mimics the effect of alfalfa CycMs2, operably under the control of a nodule-specific promoter sequence.

Preferred nodule-specific promoter sequences according to this embodiment of the present invention are listed in Table 1. Additional promoters that are suited for this purpose include the hemoglobin gene promoters derived from *Frankia spp., A. thaliana* or other plants.

In a further preferred embodiment of the present invention, the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B, in particular that mimics the effect of alfalfa CycMs2, is expressed in one of the specialised minority of plant tissues in which the activation of cell cycle progression that is generally contributed by cytokinin is in part performed by other hormones. An example of such a tissue is the youngest stem internode of cereal plants in which gibberellic acid stimulates cell division.

Accordingly, the present invention preferably provides a method of advancing cell division in the intercalary meristem of the youngest stem internode to produce greater elongation of the stem and/or to generate a more extensive photosynthetic canopy of a plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a substrate or modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a meristem specific promoter sequence.

Without being bound by any theory or mode of action, the shortened G2 phase and/or shortened G2/M phase transition in the intercalary meristem of the youngest stem internode as a consequence of increased cyclin B activity therein results in the production of a more extensive canopy. It is proposed that this leads to an increase in the plant's capacity to support grain production. The stimulatory effect of gibberellic acid application is thus obtained without side effects on flowering time and seed germination.

Preferred promoters for use according to this embodiment of the invention include meristem promoters listed in Table 1 and in particular the Proliferating Cell Nuclear Antigen (PCNA) promoter of rice described by Kosugi *et al* (1991).

In each of the preceding embodiments of the present invention, the cyclin protein is expressed under the operable control of a regulatable promoter sequence. As will be known those skilled in the art, this is generally achieved by introducing a gene

construct or vector into plant cells by transformation or transfection means. The nucleic acid molecule or a gene construct comprising same may be introduced into a cell using any known method for the transfection or transformation of said cell. Wherein a cell is transformed by the gene construct of the invention, a whole organism may be regenerated from a single transformed cell, using any method known to those skilled in the art.

By "transfect" is meant that the gene construct or vector or an active fragment thereof comprising a cyclin B gene, in particular the *CycMs2* gene, operably under the control of the regulatable promoter sequence is introduced into said cell without integration into the cell's genome.

By "transform" is meant that the gene construct or vector or an active fragment thereof comprising a cyclin B gene, in particular the *CycMs2* gene, operably under the control of the regulatable promoter sequence is stably integrated into the genome of the cell.

Accordingly, in a further preferred embodiment, the present invention provides a method of modifying one or more plant morphological and/or biochemical and/or physiological characteristics comprising

(i) introducing to a plant cell, tissue or organ a gene construct or vector comprising a nucleotide sequence that encodes a cyclin protein, such as, for example, a cyclin B protein, and in particular, the CycMs2Cdc25 protein, or a homologue, analogue or derivative thereof, operably in connection with a regulatable promoter sequence selected from the list comprising cell-specific promoter sequences, tissue-specific promoter sequences, inducible promoter sequences, organ-specific promoter sequences and cell cycle gene promoter sequences to produce a transformed or transfected cell; and

(ii) expressing said cyclin protein in one or more of said cells, tissues or organs of the plant.

In an alternative embodiment, the inventive method comprises regenerating a whole plant from the transformed cell.

Means for introducing recombinant DNA into plant tissue or cells include, but are not limited to, transformation using $CaCl_2$ and variations thereof, in particular the method described by Hanahan (1983), direct DNA uptake into protoplasts (Krens *et al*, 1982; Paszkowski *et al*, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al*, 1990) microparticle bombardment, electroporation (Fromm *et al.,* 1985), microinjection of DNA (Crossway *et al.*, 1986), microparticle bombardment of tissue explants or cells (Christou *et al*, 1988; Sanford, 1988), vacuum-infiltration of tissue with nucleic acid, or in the case of plants, T-DNA-mediated transfer from *Agrobacterium* to the plant tissue as described essentially by An *et al.*(1985), Herrera-Estrella *et al.* (1983a, 1983b, 1985).

For microparticle bombardment of cells, a microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and Wolf (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the gene construct may incorporate a plasmid capable of replicating in the cell to be transformed.

Examples of microparticles suitable for use in such systems include 1 to 5 μm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

A whole plant may be regenerated from the transformed or transfected cell, in accordance with procedures well known in the art. Plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a gene construct of the present invention and a whole plant regenerated

therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

The term "organogenesis", as used herein, means a process by which shoots and roots are developed sequentially from meristematic centres.

The term "embryogenesis", as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes.

Preferably, the transformed plants are produced by a method that does not require the application of exogenous cytokinin and/or gibberellin during the tissue culture phase, such as, for example, an *in planta* transformation method. In a particularly preferred embodiment, plants are transformed by an *in planta* method using *Agrobacterium tumefaciens* such as that described by Bechtold *et al.*, (1993) or Clough *et al* (1998), wherein *A. tumefaciens* is applied to the outside of the developing flower bud and the binary vector DNA is then introduced to the developing microspore and/or macrospore and/or the developing seed, so as to produce a transformed seed without the exogenous application of cytokinin and/or gibberellin. Those skilled in the art will be aware that the selection of tissue for use in such a procedure may vary, however it is preferable generally to use plant material at the zygote formation stage for *in planta* transformation procedures.

The regenerated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give homozygous second

generation (or T2) transformant, and the T2 plants further propagated through classical breeding techniques.

The regenerated transformed organisms contemplated herein may take a variety of forms. For example, they may be chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., in plants, a transformed root stock grafted to an untransformed scion).

A further aspect of the present invention clearly provides the gene constructs and vectors designed to facilitate the introduction and/or expression and/or maintenance of the cyclin protein-encoding sequence and regulatable promoter into a plant cell, tissue or organ.

In addition to the cyclin protein-encoding sequence and regulatable promoter sequence, the gene construct of the present invention may further comprise one or more terminator sequences.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3′-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3′-end of a primary transcript. Terminators active in cells derived from viruses, yeasts, moulds, bacteria, insects, birds, mammals and plants are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants.

Examples of terminators particularly suitable for use in the gene constructs of the present invention include the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene terminator, the *Agrobacterium tumefaciens* octopine synthase (OCS) gene

terminator sequence, the Cauliflower mosaic virus (CaMV) 35S gene terminator sequence, the *Oryza sativa* ADP-glucose pyrophosphorylase terminator sequence (t3'Bt2), the *Zea mays zein* gene terminator sequence, the *rbcs-1A* gene terminator, and the *rbcs-3A* gene terminator sequences, amongst others.

Those skilled in the art will be aware of additional promoter sequences and terminator sequences which may be suitable for use in performing the invention. Such sequences may readily be used without any undue experimentation.

The gene constructs of the invention may further include an origin of replication sequence which is required for maintenance and/or replication in a specific cell type, for example a bacterial cell, when said gene construct is required to be maintained as an episomal genetic element (eg. plasmid or cosmid molecule) in said cell.

Preferred origins of replication include, but are not limited to, the *f1*-ori and *col*E1 origins of replication.

The gene construct may further comprise a selectable marker gene or genes that are functional in a cell into which said gene construct is introduced.

As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a gene construct of the invention or a derivative thereof.

Suitable selectable marker genes contemplated herein include the ampicillin resistance (Amp^r), tetracycline resistance gene (Tc^r), bacterial kanamycin resistance gene (Kan^r), phosphinothricin resistance gene, neomycin phosphotransferase gene (*npt*II), hygromycin resistance gene, β-glucuronidase (GUS) gene, chloramphenicol

acetyltransferase (CAT) gene, green fluorescent protein (*gfp*) gene (Haseloff *et al*, 1997), and luciferase gene, amongst others.

A further aspect of the invention clearly extends to a plant cell, tissue, organ or whole plant that has been transformed or transfected with an isolated nucleic acid molecule that comprises a nucleotide sequence which encodes a cyclin protein, wherein the expression of said nucleotide sequence is placed operably under the control of a plant-expressible cell-specific promoter sequence, plant-expressible tissue-specific promoter sequence, a plant-expressible organ-specific promoter sequence, a plant-expressible cell cycle specific gene promoter, or alternatively, a plant-expressible constitutive promoter sequence such that said plant-expressible constitutive promoter sequence and said nucleotide sequence encoding a cyclin protein are integrated into a transposable genetic element.

The present invention is applicable to any plant, in particular a monocotyledonous plants and dicotyledonous plants including a fodder or forage legume, ornamental plant, food crop, tree, or shrub selected from the list comprising *Acacia spp., Acer spp., Actinidia spp.,Aesculus spp., Agathis australis, Albizia amara, Alsophila tricolor, Andropogon spp., Arachis spp, Areca catechu, Astelia fragrans, Astragalus cicer, Baikiaea plurijuga, Betula spp., Brassica spp., Bruguiera gymnorrhiza, Burkea africana, Butea frondosa, Cadaba farinosa, Calliandra spp, Camellia sinensis, Canna indica, Capsicum spp., Cassia spp., Centroema pubescens, Chaenomeles spp.,Cinnamomum cassia, Coffea arabica, Colophospermum mopane, Coronillia varia, Cotoneaster serotina, Crataegus spp., Cucumis spp., Cupressus spp., Cyathea dealbata, Cydonia oblonga, Cryptomeria japonica, Cymbopogon spp., Cynthea dealbata, Cydonia oblonga, Dalbergia monetaria, Davallia divaricata, Desmodium spp., Dicksonia squarosa, Diheteropogon amplectens, Dioclea spp, Dolichos spp., Dorycnium rectum, Echinochloa pyramidalis, Ehrartia spp., Eleusine coracana, Eragrestis spp., Erythrina spp., Eucalyptus spp., Euclea schimperi, Eulalia villosa, Fagopyrum spp., Feijoa sellowiana, Fragaria spp., Flemingia spp, Freycinetia banksii, Geranium thunbergii,*

Ginkgo biloba, Glycine javanica, Gliricidia spp, Gossypium hirsutum, Grevillea spp., Guibourtia coleosperma, Hedysarum spp., Hemarthia altissima, Heteropogon contortus, Hordeum vulgare, Hyparrhenia rufa, Hypericum erectum, Hyperthelia dissoluta, Indigo incarnata, Iris spp., Leptarrhena pyrolifolia, Lespediza spp., Lettuca spp., Leucaena leucocephala, Loudetia simplex, Lotonus bainesii, Lotus spp., Macrotyloma axillare, Malus spp., Manihot esculenta, Medicago sativa, Metasequoia glyptostroboides, Musa sapientum, Nicotianum spp., Onobrychis spp., Ornithopus spp., Oryza spp., Peltophorum africanum, Pennisetum spp., Persea gratissima, Petunia spp., Phaseolus spp., Phoenix canariensis, Phormium cookianum, Photinia spp., Picea glauca, Pinus spp., Pisum sativum, Podocarpus totara, Pogonarthria fleckii, Pogonarthria squarrosa, Populus spp., Prosopis cineraria, Pseudotsuga menziesii, Pterolobium stellatum, Pyrus communis, Quercus spp., Rhaphiolepsis umbellata, Rhopalostylis sapida, Rhus natalensis, Ribes grossularia, Ribes spp., Robinia pseudoacacia, Rosa spp., Rubus spp., Salix spp., Schyzachyrium sanguineum, Sciadopitys verticillata, Sequoia sempervirens, Sequoiadendron giganteum, Sorghum bicolor, Spinacia spp., Sporobolus fimbriatus, Stiburus alopecuroides, Stylosanthos humilis, Tadehagi spp, Taxodium distichum, Themeda triandra, Trifolium spp., Triticum spp., Tsuga heterophylla, Vaccinium spp., Vicia spp.Vitis vinifera, Watsonia pyramidata, Zantedeschia aethiopica, Zea mays, amaranth, artichoke, asparagus, broccoli, brussel sprout, cabbage, canola, carrot, cauliflower, celery, collard greens, flax, kale, lentil, oilseed rape, okra, onion, potato, rice, soybean, straw, sugarbeet, sugar cane, sunflower, tomato, squash, and tea, amongst others, or the seeds of any plant specifically named above or a tissue, cell or organ culture of any of the above species.

Accordingly, the present invention clearly extends to any plant produced by the inventive method described herein, and any and all plant parts and propagules thereof. The present invention extends further to encompass the progeny derived from a primary transformed or transfected cell, tissue, organ or whole plant that has been produced by the inventive method, the only requirement being that said progeny exhibits the same genotypic and/or phenotypic characteristic(s) as that (those)

characteristic(s) that has (have) been produced in the parent by the performance of the inventive method.

By "genotypic characteristic" is meant the composition of the genome and, more particularly, the introduced gene encoding the cyclin protein.

By "phenotypic characteristic" is meant one or more plant morphological characteristics and/or plant biochemical characteristics and/or plant physiological characteristics that are produced by ectopic expression of a cyclin protein in a plant.

Preferably, the plant is produced according to the inventive method is transfected or transformed with a genetic sequence, or amenable to the introduction of a protein, by any art-recognised means, such as microprojectile bombardment, microinjection, *Agrobacterium*-mediated transformation (including *in planta* transformation), protoplast fusion, or electroporation, amongst others.

The present invention is further described with reference to the following non-limiting Examples and to the drawings.

EXAMPLE 1

Regulatable ectopic expression of alfalfa CycMs2 mitotic cyclin in tobacco

The cycMs2 alfalfa mitotic cyclin was expressed as a fusion protein with haemaglutinin (HA) in tobacco plants under the control of tetracycline-regulatable promoter construct. To facilitate the detection of the transgene, the haemaglutinin epitope tag (HA) was fused to the C-terminus of the CycMs2 coding region, to produce the fusion designated as "CycMs2-HA".

After *Agrobacterium*-mediated transformation, the expression of CycMs2-HA was determined in leaves from 5 independent transgenic lines treated with 1 mg/l Cl-

tetracyclin for 24 h (Figure 1-1). In all of these transgenic lines, the mRNA of cycMs2-HA transgene could be detected by RNA-blot hybridisation. In 4 lines, the expression was strictly dependent on the addition of tetracycline.

A suspension culture was initiated on 2,4-D-containing medium, using the line 2 plant (Figure 1-1) as starting material. The expression of cycMs2-HA was still strictly dependent on tetracycline in cultured cells and the accumulation of cycMs2-HA mRNA could already be detected after 10 minutes of incubation with 1 mg/l Cl-tetracycline (Figure 1-2).

To determine the optimal tetracycline concentration for expression of the CycMs2-HA transgene, cultured cells were treated with a range of Cl-tetracycline concentrations for 24 h, and the expression of the CycMs2-HA protein was detected by protein blotting with the HA antibody (Figure 1-3, upper panel). A sharp increase in the production of CycMs2-HA protein was found at 0.01 mg/l Cl-tetracycline concentration.

To determine whether the ectopically-expressed CycMs2-HA protein forms an active complex with cyclin-dependent kinase (CDK), we immunopurified the complex from cell extract treated with different tetracycline concentrations and measured the protein kinase activity of the CDK. The increase in activity of CycMs2-HA complexed with CDK was correlated with the amount of expressed cycMs2-HA cyclin protein in these extracts, indicating that the amount of cyclin is rate-limiting to produce active CDK complexes (Figure 1-3, middle panel). When different CDK complexes were isolated by the binding to the $p13^{suc1}$-protein, CDK activities were comparable in cells expressing or not expressing the CycMs2-HA protein, indicating that the CycMs2-HA associated CDK activity is only a minor portion of the total CDK activity in these extracts (Figure 1-3, lower panel).

EXAMPLE 2

The CycMs2-mitotic cyclin protein is in the nucleus

By indirect immunofluorescence microscopy using anti-HA antibody as a probe, the localisation of the ectopically-expressed CycMs2-HA protein was determined (Figure 2). In the presence of tetracycline, the CycMs2-HA protein was detected in the nucleus of around 60% of cells (Figures 2-1 and 2-2). No signal was detectable without incubation of cells in tetracycline (Figures 2-3 and 2-4).

To confirm the nuclear localisation by other means, cells were fractionated into cytoplasm and nucleus and the activity of the CycMs2-HA associated CDK was determined by measuring the protein kinase activity of the complex immunoprecipitated from nuclear and cytoplasmic extracts. The majority of CycMs2-HA associated kinase activity was present in the nucleus (Figure 3).

The intracellular localisation of the CycMs2 in tobacco cells was also determined using GFP and GFP-protein fusion genes placed in a regulated plant expression vector pBIN-HygTX. In the binary vector pBIN-HygTX the expression is directed by a modified cauliflower mosaic virus (CaMV) 35S promoter combined with the regulatable tetracyclin expression system (Weinmann *et al*, Plant J. 1994 Apr;5(4):559-69; Gatz *et al* at 1992, Plant J 2(3), 397-404). A modified version of GFP with s65T mutation and altered codon usage was used (Sheen *et al* 1995, Plant J 8(5) 777-784). Figure 10 indicates that the CycMs2-GFP fusion is constitutively localised to the nucleus and absent in cells in telophase (arrow). Epifluorescence microphotograph of GFP fluorescence (A); and DIC phase contrast image of the cell (B).

EXAMPLE 3

The length of the G2 phase is shortened in cells that ectopically express the CycMs2-HA mitotic cyclin

Having determined that the amount of cyclin is rate limiting to form an active CDK

complex in plant cells, we asked if the ectopic expression of CycMs2 mitotic cyclin in G2 cells is sufficient to enter mitosis.

Cell divisions were synchronised by releasing cells from a block by the inhibition of DNA synthesis with aphidicolin in cultured cells. The expression of CycMs2-HA protein was induced by adding 0.1 mg/l Cl-tetracycline, after removing aphidicolin, and the progression of cell cycle was followed by measuring the DNA content of cells with flow cytometry (Pfosser *et al.*, 1995), counting the number of mitotic cells and counting the mitosis-specific microtubule structures (Figure 4).

In tetracycline-treated cells, the number of mitotic divisions started to increase 8 h after aphidicolin release and the maximal number of mitotic divisions were found at 10 h (Figure 5A). In contrast, cells incubated without tetracycline entered mitosis 2 hours later, and the highest number of mitotic divisions was achieved at 12 h. A similar 2 hour advance in the formation of mitotic microtubule structures were observed in cells ectopically expressing the mitotic CycMs2-HA cyclin protein in the presence of tetracyclin.

The measurement of DNA content by flow cytometry further confirmed these results (Figure 4-3).

While at 8 h a similar distribution of nuclei with G2 and G1 DNA contents was found in tetracycline-treated and control cells, at 10 h in the presence of tetracycline a high proportion of cells already had nuclei with G1 DNA content, indicating that they passed through mitosis.

From these experiments we can conclude that the ectopic expression of cycMs2 mitotic cyclin in G2 cells does not induce mitosis directly but advances cells to enter mitosis 2 hours earlier (Figure 5-4).

As independent molecular proof for the advancement of cell divisions in the cells ectopically expressing the CycMs2-HA protein in G2 phase, mRNA was prepared from these cells and hybridised with an HA fragment to detect the transgene (Figure 5). Additionally, the expression of S phase and mitosis-specific marker genes were also monitored, in particular the histone H4 and an endogenous mitotic tobacco cyclin (cycM) as shown in Figure 5.

Data shown in Figure 5 indicate that histone H4 mRNA was similarily present in samples with aphidicolin and at 3 h both with and without tetracycline, however at 20 h in the presence of tetracycline a higher level of histone H4 expression was observed than that in the control samples, indicating that some cells reached the second S phase at 20 h when the mitotic cyclin is ectopically expressed. The expression of the tobacco cycM mitotic cyclin followed the number of mitotic cells during the synchronous cell division and was similarily advanced by about 2 hours in the presence of tetracycline.

EXAMPLE 4

Ectopic expression of CycMs2 mitotic cyclin inhibits root regeneration in culture from tobbaco leaves

Regeneration of shoots and roots from tobacco leaves is dependent on the auxin/cytokinin ratio: high auxin to cytokinin concentration favours root regeneration, while high cytokinin to auxin concentration favours shoot regeneration.

In *in vitro* regeneration experiments, with leaf disks of the transgenic line containing the tetracycline inducible cycMs2 gene, the regeneration of shoots still required cytokinin even in the presence of ectopic mitotic cyclin expression, but root formation was inhibited by ectopic cyclin expression (Figure 6).

We measured the DNA content of nuclei from leaves treated for 5 days with different NAA and BAP concentrations in the presence or absence of tetracyclin (Figure 7). A

higher percentage of cells with G2 DNA content was found in leaf cells from wild type plants treated with high auxin to cytokinin ratio favouring root formation than in leaves treated with high cytokinin to auxin ratio, favouring shoot regeneration. This observation is compatible with the notion that cells spend more time in G2 when cultured on medium favouring root formation compared to the treatment favouring shoot formation. In leaf disks from transgenic tobacco plants ectopically expressing cycMs2 the number of cells in G2 was reduced indicating a shortened G2 phase.

EXAMPLE 5

DISCUSSION

Plants are mutlicellular organisms with defined shapes and sizes. A developmentally determined body plan is elaborated by the controlled timing and orientation of cell divisions in restricted zones called meristems. Conserved regulators of cell division in eukaryotes are the cyclin-dependent protein kinases (CDKs). The expression of cyclin genes are tightly regulated in plants, e.g. B-type cyclins were only found in mitotic cells. We used a tetracycline regulatable promoter construct to study if cyclin expression is limiting in the timing of cell divisions. The expression of a mitotic cyclin, the alfalfa cycMs2, results in an elevated CDK activity, and cells with increased cyclin amount entered mitosis earlier. We tested the consequences of a shortened G2 phase on the auxin- and cytokinin-dependent regeneration of shoots and roots from leaf disks. A premature passage through mitosis inhibits root formation, thus having a cytokinin-like effect.

We propose a model, shown in Figure 8, that in which shoot versus root regeneration is regulated by the G1 and G2 exit points from the cell cycle. According to our model, regeneration of roots and root development and/or initiation depends upon whether a cell exits the cell cycle in the G1 or G2 phase. Roots are formed when cells exit the cell cycle in G2 phase, while shoot formation is dependent on an exit from G1 phase or following a shortened G2 phase.

EXAMPLE 5

Expression of CycMs2 under the control of the patatin gene promoter increases tuber size and number in potato plants

The CycMs2 coding sequence is cloned between the promoter of a class I patatin gene (Liu *et al.,*1991) and the transcription termination signals of the nopaline synthase (NOS) gene of *Agrobacterium tumefaciens*. Preferentially, the B repeat region and the distal region of the A repeat of the patatin promoter is used, without the proximal region of the A repeat. The proximal region of the A repeat of the patatin promoter confers sucrose-responsiveness in various tissues, which is not a desirable characteristic for our purposes (Grierson *et al.,* 1994). This construct is placed in a binary vector, mobilized to *Agrobacterium tumefaciens*, and the introduced into potato plants.

The CycMs2 protein is expressed under the control of the Class I patatin promoter when the first stolon starts to tuberize, consistent with the expression pattern for the patatin gene (Liu *et al.,* 1991). At this stage, expression is associated with both internal and external phloem. After tuber induction has occurred, promoter activity is found both in tuberized stolons and in non-tuberized stolons. Expression then expands to the entire storage parenchyma, cortex and pith, but remains absent from the periderm.

Because the Class II patatin promoters are expressed in the periderm and as such are complementary to the Class I promoters (Köster-Töpfer *et al.,*; Liu *et al.,* 1991; Nap *et al.,* 1992), it is beneficial to have CycMs2 expression driven by both Class I and Class II promoters within the same plant. Because the Class I patatin promoter is not expressed before the first stolon initiates tuberization, no effects of Class I patatin-CycMs2 transgenes is seen on tuber initiation. However, the Class I patatin promoter drives CycMs2 expression very early after tuber initiation onwards, allowing a maximal impact of CycMs2 activity on organ formation and, as a consequence, on tuber size. The fact that the Class I patatin promoter activity subsequently also appears in non-tuberized stolons implies that the Class I patatin – CycMs2 transgene increases both

the size and number of tubers.

EXAMPLE 6

Expression of CycMs2 under the control of endosperm-specific promoters increases grain size and yield of grain crop plants

The alfalfa CycMs2 coding sequence is placed operably in connection with the endosperm-specific *ltr1* promoter from barley, or a synthetic promoter containing the endosperm box (GCN motif) of the barley *Hor2* gene (Vicente-Carbajosa *et al.,* 1998). In each case, the CycMs2 structural gene is placed upstream of the transcription termination signals of the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene. Cereals, in particular rice, maize, wheat and barley, are transformed using standard procedures, in particular microprojectile bombardment or *Agrobacterium*-mediated transformation systems, with the gene constructs.

The grain size and starch storage capacity of the endosperm of the seeds of transformed plants is increased relative to otherwise isogenic non-transformed plants.

EXAMPLE 7

Expression of CycMs2 under the control of the *cab-6* or *ubi7* promoters reduces leaf necrosis and chlorosis in lettuce plants

The alfalfa CycMs2 coding sequence is placed operably in connection with the leaf-specific *cab-6* gene promoter derived from *Pinus* (Yamamoto *et al.,* 1994) or senescence-specific *ubi7* gene promoter (Garbarino *et al.,* 1995). In each case, the CycMs2 structural gene is placed upstream of the transcription termination signals of the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene. Lettuce is transformed as described by Bechtold *et al.,* 1993.

Leaf deterioration (chlorosis and necrosis) in lettuce, for example as a consequence of post-harvest storage, is delayed in transformed lettuce plants compared to non-

transformed control plants.

EXAMPLE 8

Ectopic expression of the CycMs2 (Medsa CycB2;2) mitotic cyclin mimics cytokinin effects in dark-grown seedlings

Tobacco seedlings of CycMs2 T2 transformants (TM100 2/5) and transformants with a control plasmid (pBin-HygTX) (see Example 1 for construct and transformation details) were germinated and grown in the dark for 14 days and then placed on light. Photographs were taken 18 and 24 days after germination (see Figure 9). The TM100 2/5 seedlings show a retardation in growth (fresh weight) of approximately 20-30 % in comparison to the controls. The roots of the TM100 2/5 seedlings show more branching, there is reduced root length and hypocotyl elongation is strongly retarded in comparison to controls.

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WE CLAIM:

1. A method of modifying cell fate or development, or one or more plant morphological and/or biochemical and/or physiological characteristics comprising expressing in one or more particular cells, tissues or organs of a plant, an isolated nucleic acid molecule comprising a nucleotide sequence that encodes a cyclin protein or a homologue, analogue or derivative thereof operably under the control of a regulatable promoter sequence that is operable in a plant or a cell, tissue or organ thereof.

2. The method according to claim 1, wherein the cyclin protein is a cyclin B protein or a homologue, analogue or derivative thereof.

3. The method according to claim 2, wherein the cyclin B protein is derived from a plant.

4. The method according to claim 3 wherein the plant from which the cyclin B protein is derived is a dicotyledonous plant.

5. The method according to claim 4, wherein the cyclin B protein is the alfalfa CycMs2 mitotic cyclin or a homologue, analogue, or derivative thereof.

6. The method according to claim 1, wherein the cyclin protein is a cyclin B substrate or a modified cyclin B substrate that is functionally equivalent to a cyclin B protein.

7. The method according to claim 6, wherein the cyclin B substrate is CDK or a modified form thereof.

8. The method according to claim 1, wherein the regulatable promoter is a tetracycline-inducible promoter sequence.

9. The method according to claim 1 wherein the modified cell fate cell fate or development, or one or more plant morphological and/or biochemical and/or physiological characteristics comprises shortening the duration of the G2 phase of the cell cycle.

10. The method according to claim 1 wherein the modified cell fate cell fate or development, or one or more plant morphological and/or biochemical and/or physiological characteristics comprises shortening the G2/M phase transition of a cell.

11. The method according to claim 9 or 10 wherein the cyclin protein is co-expressed with one or more cyclin B substrates and/or one or more modified cyclin B substrates or with another synergistic or non-antagonistic cell cycle control protein.

12. The method according to claim 1 wherein modifying cell fate cell fate or development, or one or more plant morphological and/or biochemical and/or physiological characteristics comprises advancing cell division in a plant cell, tissue or organ.

13. The method according to claim 1 wherein the plant morphological and/or biochemical and/or physiological characteristic is regulated by the sink/source relationships in the plant or a cell, tissue or organ thereof.

14. The method according to claim 1 wherein modifying cell fate or development comprises reducing or inhibiting or delaying root development in the plant.

15. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic is selected from the group consisting of: (i) enhanced seed set; (ii) enhanced seed size; (iii) enhanced grain yield; and (iv) enhanced endoreduplication in the seed of the plant, and wherein the regulatable promoter sequence is at least operable in the seed of a plant or a cell, tissue or organ of said seed.

16. The method according to claim 15, wherein the regulatable promoter sequence is selected from the group consisting of: (i) a barley *Amy32b* gene promoter sequence; (ii) a Cathepsin β-like gene promoter sequence; (iii) a wheat ADP-glucose pyrophosphorylase gene promoter sequence; (iv) a maize zein gene promoter sequence; (v) a rice glutelin gene promoter sequence; (vi) a legumin gene promoter sequence; (vii) a *napA* gene promoter sequence; (viii) a Brazil Nut albumin gene promoter sequence; (ix) a pea vicilin gene promoter sequence; (x) a sunflower oleosin gene promoter sequence; (xi) a barley *ltr1* gene promoter sequence; and (xii) a barley *Hor2* gene promoter sequence.

17. The method according to claim 15, wherein the regulatable promoter sequence is operable in the endosperm of the seed.

18. The method according to claim 17, wherein the regulatable promoter sequence comprises a rice prolamin *NRP33* promoter sequence.

19. The method according to claim 17, wherein the regulatable promoter sequence comprises a synthetic promoter that contains a rice *REB* gene promoter sequence.

20. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic is selected from the group consisting of: enhanced tuber formation and enhanced tuber development, and

wherein the regulatable promoter sequence is at least operable in the tuber of a plant or a cell, or tissue of said tuber.

21. The method according to claim 20, wherein the plant is potato.

22. The method according to claim 20 wherein the promoter sequence is a potato patatin gene promoter sequence.

23. The method according to claim 22, wherein the patatin gene promoter sequence is selected from the group consisting of: (i) a class I patatin gene promoter sequence; and (ii) a class II patatin gene promoter sequence.

24. The method according to claim 23, wherein the class I patatin gene promoter sequence has a reduced number of functional sucrose-responsive elements compared to the naturally-occurring class I patatin gene from which said promoter sequence was derived.

25. The method according to claim 24 wherein the number of functional sucrose-responsive elements is reduced by deletion of a proximal region of the A repeat in said class I patatin gene.

26. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises reduced or delayed chlorosis and/or necrosis of the green leaf tissue of the plant, and wherein the regulatable promoter sequence is at least operable in the leaf of a plant or a cell, or tissue of said leaf.

27. The method according to claim 26, wherein the promoter is selected from the

group consisting of: (i) a SAM22 gene promoter sequence; (ii) a *rbcs-1A* gene promoter sequence; (iii) a *rbcs-3A* gene promoter sequence; (iv) a *cab-6* gene promoter sequence; and (v) a *ubi7* gene promoter sequence.

28. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises modified sink/source relationships of a plant tissue, organ or whole plant.

29. The method according to claim 24 wherein the regulatable promoter sequence is a tetracycline-inducible promoter sequence.

30. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises enhanced bushiness or reduced apical dominance of the plant, and wherein the regulatable promoter sequence is at least operable in the meristem of a plant or a meristem cell.

31. The method according to claim 30 wherein the meristem is a lateral meristem.

32. The method according to claim 30 wherein the meristem is an apical meristem.

33. The method according to claim 30 wherein the regulatable promoter sequence is selected from the group consisting of: (i) a *LEAFY* gene promoter sequence; (ii) a *knat1* gene promoter sequence; (iii) a *kn1* gene promoter sequence; and (iv) a *CLAVATA1* gene promoter sequence.

34. The method according to claim 30 wherein the reduced apical dominance comprises reduced or inhibited root apical dominance or reduced or inhibited root development.

35. The method according to claim 30 wherein the formation of lateral roots is modified.

36. The method according to claim 34, wherein the promoter sequence is the tobacco auxin-inducible gene promoter.

37. The method according to claim 35, wherein the promoter sequence is the tobacco auxin-inducible gene promoter.

38. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises enhanced nitrogen fixing capacity of the plant or a nodule of said plant, and wherein the regulatable promoter sequence is at least operable in the nodule of a plant or a cell, or tissue of said nodule.

39. The method according to claim 38, wherein the regulatable promoter sequence is selected from the group consisting of: (i) a *nif* gene promoter sequence; (ii) a *nifH* gene promoter sequence; (iii) a ENOD gene promoter sequence; (iv) a PEPC gene promoter sequence; (v) a leghaemoglobin gene promoter sequence; and (vi) a hemoglobin gene promoter sequence.

40. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic is selected from the group consisting of: enhanced strength, enhanced stem thickness, enhanced stability, and enhanced wind-resistance, and wherein the regulatable promoter sequence is at least operable in the stem of a plant or a cell, or tissue thereof.

41. The method according to claim 40, wherein the promoter sequence is selected

from the group consisting of: (i) a *rbcs-1A* gene promoter sequence; (ii) a *rbcs-3A* gene promoter sequence; (iii) a *AtPRP4* gene promoter sequence; (iv) a *T. bacilliform* virus gene promoter sequence; and (v) a sucrose-binding protein gene promoter sequence.

42. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises modified lignin content, and wherein the regulatable promoter sequence is at least operable in the cambium or vasculature of a woody plant, or a cell, tissue or organ of said cambium or vasculature.

43. The method according to claim 42, wherein the regulatable promoter sequence is selected from the group consisting of: (i) a cinnamoyl alcohol dehydrogenase (CAD) gene promoter sequence; (ii) a laccase gene promoter sequence; (iii) a cellulose synthase gene promoter sequence; and (iv) a xyloglucan endotransglucosylase (XET) gene promoter sequence.

44. The method according to claim 42, wherein the regulatable promoter sequence is the auxin-inducible SAUR promoter sequence.

45. The method according to claim 42, wherein the regulatable promoter sequence is the *rolB* promoter sequence.

46. The method according to claim 42, wherein the woody plant is selected from the group consisting of: *Eucalyptus spp.; Populus spp.; Quercus spp.; Acer spp.; Juglans spp.; Fagus spp.; Acacia spp.;* and teak.

47. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises partial or complete

inhibition of the arrest of DNA replication in a plant cell under growth-limiting conditions.

48. The method according to claim 47 wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises enhanced endoreplication and/or enhanced endoreduplication.

49. The method according to claim 1, wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises enhanced cell expansion.

50. The method according to claim 1, wherein the regulatable promoter sequence comprises a regulatable cell-specific promoter sequence.

51. The method according to claim 1, wherein the regulatable promoter sequence comprises a regulatable tissue-specific promoter sequence.

52. The method according to claim 51, wherein the tissue-specific promoter sequence is selected from the group consisting of:(i) a phloem-specific promoter sequence; (ii) a cell-wall-specific promoter sequence; (iii) a root cortex-specific promoter sequence; (iv) a root vasculature-specific promoter sequence; (v) a tapetum-specific promoter sequence; and (vi) a meristem-specific promoter sequence.

53. The method according to claim 1, wherein the regulatable promoter sequence comprises a regulatable organ-specific promoter sequence.

54. The method according to claim 53, wherein the regulatable promoter sequence is selected from the group consisting of: (i) an aleurone-specific promoter sequence;

(ii) a flower-specific promoter sequence; (iii) a fruit-specific promoter sequence; (iv) a leaf-specific promoter sequence; (v) a nodule-specific promoter sequence; (vii) a pollen-specific promoter sequence; (viii) an anther-specific promoter sequence; (ix) a root-specific promoter sequence; (x) a seed-specific promoter sequence; (xi) an endosperm-specific promoter sequence; (xii) an embryo-specific promoter sequence; and (xiii) a stigma-specific promoter sequence.

55. The method according to claim 1, wherein the regulatable promoter sequence comprises a regulatable cell cycle-specific promoter sequence.

56. The method according to claim 56 wherein the regulatable cell cycle-specific promoter sequence comprises a cell cycle gene promoter sequence.

57. The method according to claim 1, wherein the nucleotide sequence encoding the cyclin protein or a homologue, analogue or derivative is expressed by a process comprising introducing a gene construct that comprises said nucleotide sequence operably in connection with the regulatable promoter sequence into a plant cell and culturing said plant cell under conditions sufficient for transcription and translation to occur.

58. The method according to claim 57, wherein culturing of the plant cell under conditions sufficient for transcription and translation to occur includes organogenesis or embryogenesis.

59. The method according to claim 59 wherein the organogenesis or embryogenesis includes regeneration of the plant cell into a whole plant.

60. A transformed plant produced by the method according to claim 59.

61. A plant part, propagule, or progeny, of the plant according to claim 60, wherein said plant part, propagule or progeny exhibits one or more modified plant morphological and/or biochemical and/or physiological characteristics of said plant as a consequence of the ectopic expression therein of a cyclin protein or a homologue, analogue or derivative of said cyclin.

62. A gene construct comprising a nucleotide sequence encoding a cyclin protein or a homologue, analogue or derivative thereof, placed operably in connection with a regulatable promoter sequence that is operable in a plant or a cell, tissue or organ of said plant, wherein said regulatable promoter sequence is selected from the group consisting of: (i) a tetracycline-inducible promoter sequence; (ii) a patatin gene promoter sequence; (iii) a modified patatin gene promoter sequence having a deletion in a sucrose-responsive element; (iv) an auxin-inducible SAUR gene promoter sequence; (v) a *rolB* gene promoter sequence; (vi) a rice prolamin *NRP33* gene promoter sequence; (vii) a synthetic promoter sequence comprising one or more endosperm box motifs derived of the barley *Hor2* gene; (viii) a *LEAFY* gene promoter sequence; (ix) a *knat1* gene promoter sequence; (x) a *kn1* gene promoter sequence; (xi) a *CLAVATA1* gene promoter sequence; (xii) a *cab-6* gene promoter sequence; (xiii) a rice REB gene promoter sequence; and (xiv) a *ubi7* gene promoter sequence.

63. A transformed plant comprising the gene construct according to claim 62, wherein said plant exhibits one or more modified plant morphological and/or biochemical and/or physiological characteristics compared to otherwise isogenic non-transformed plants, and wherein said characteristics are selected from the group consisting of: (i) enhanced stem strength; (ii) enhanced stem thickness; (iii) enhanced stem stability; (iv) enhanced wind-resistance of the stem; (v) enhanced tuber formation; (vi) enhanced tuber development; (vii) increased lignin content; (viii) enhanced seed set; (ix) enhanced seed production; (x) enhanced seed size; (xi) enhanced grain yield; (xii) enhanced ploidy of the seed; (xiii) enhanced endosperm size; (xiv) reduced apical dominance; (xv) increased bushiness; (xvi) reduced root formation; (xvii) enhanced

nitrogen-fixing capability; (xviii) enhanced nodulation or nodule size; (xix) reduced or delayed leaf chlorosis; (xx) reduced or delayed leaf necrosis; (xxi) partial or complete inhibition of the arrest of DNA replication in a plant cell under growth-limiting conditions; (xxii) enhanced endoreplication and/or enhanced endoreduplication; and (xxiii) enhanced cell expansion.

64. A plant part, propagule, or progeny, of the plant according to claim 63, wherein said plant part, propagule or progeny exhibits one or more of the modified plant morphological and/or biochemical and/or physiological characteristics of said plant as a consequence of the ectopic expression therein of a cyclin protein, or a homologue, analogue or derivative of a cyclin protein.

65. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises an extended photosynthetic canopy of a crop plant, and wherein the regulatable promoter sequence is at least operable in the internode meristem of stem tissue of said crop plant.

66. The method according to claim 65, wherein the regulatable promoter sequence is a Proliferating Cell Nuclear Antigen (PCNA) promoter of rice.

67. The method according to claim 65 wherein the yield and/or sink strength of grain is enhanced.

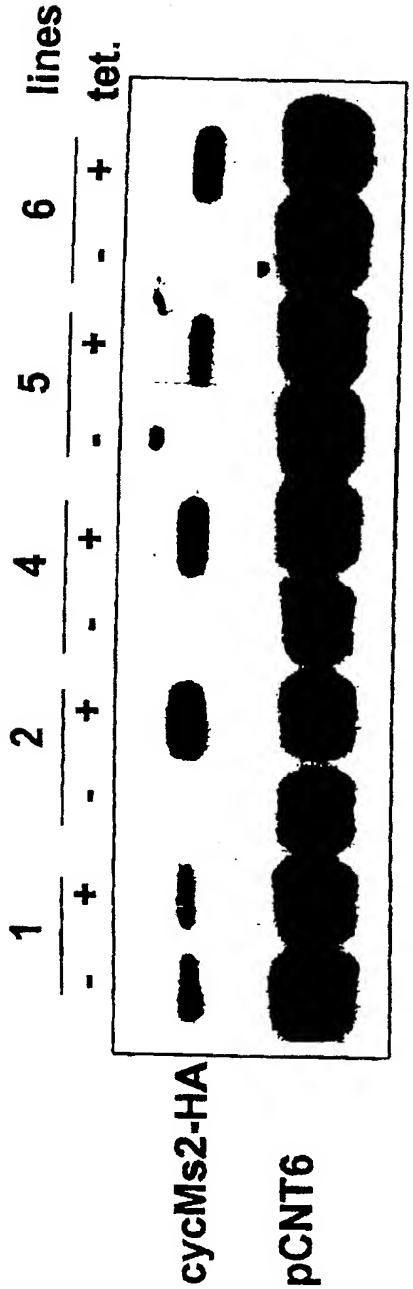


FIGURE 1A

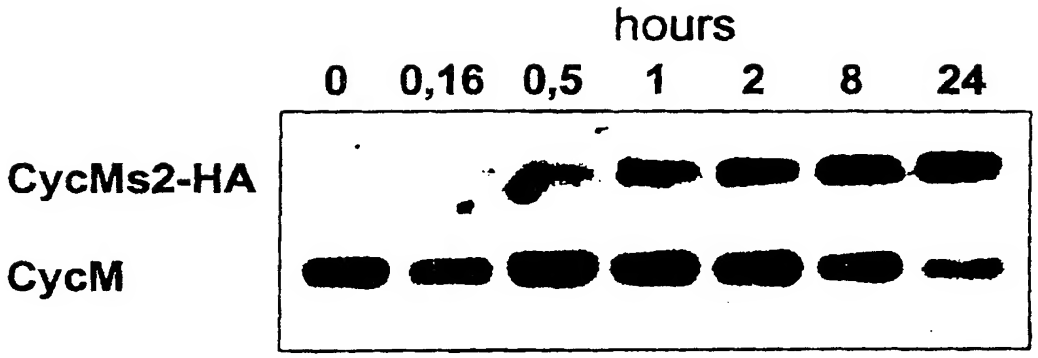
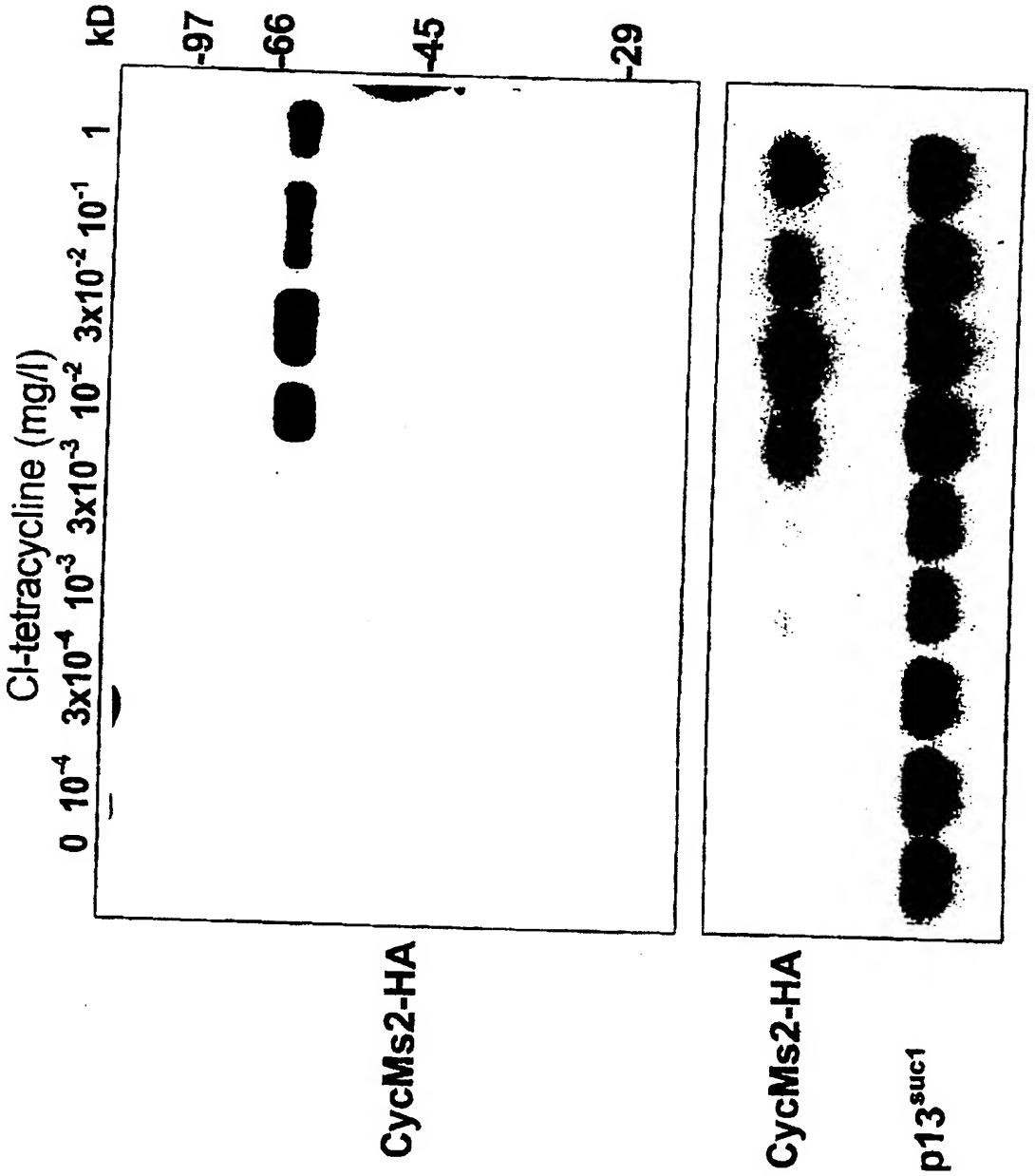


FIGURE 1B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-66
-45
-29
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10-2
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10-1
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CycMs2-HA
p13suc1

FIGURE 1C

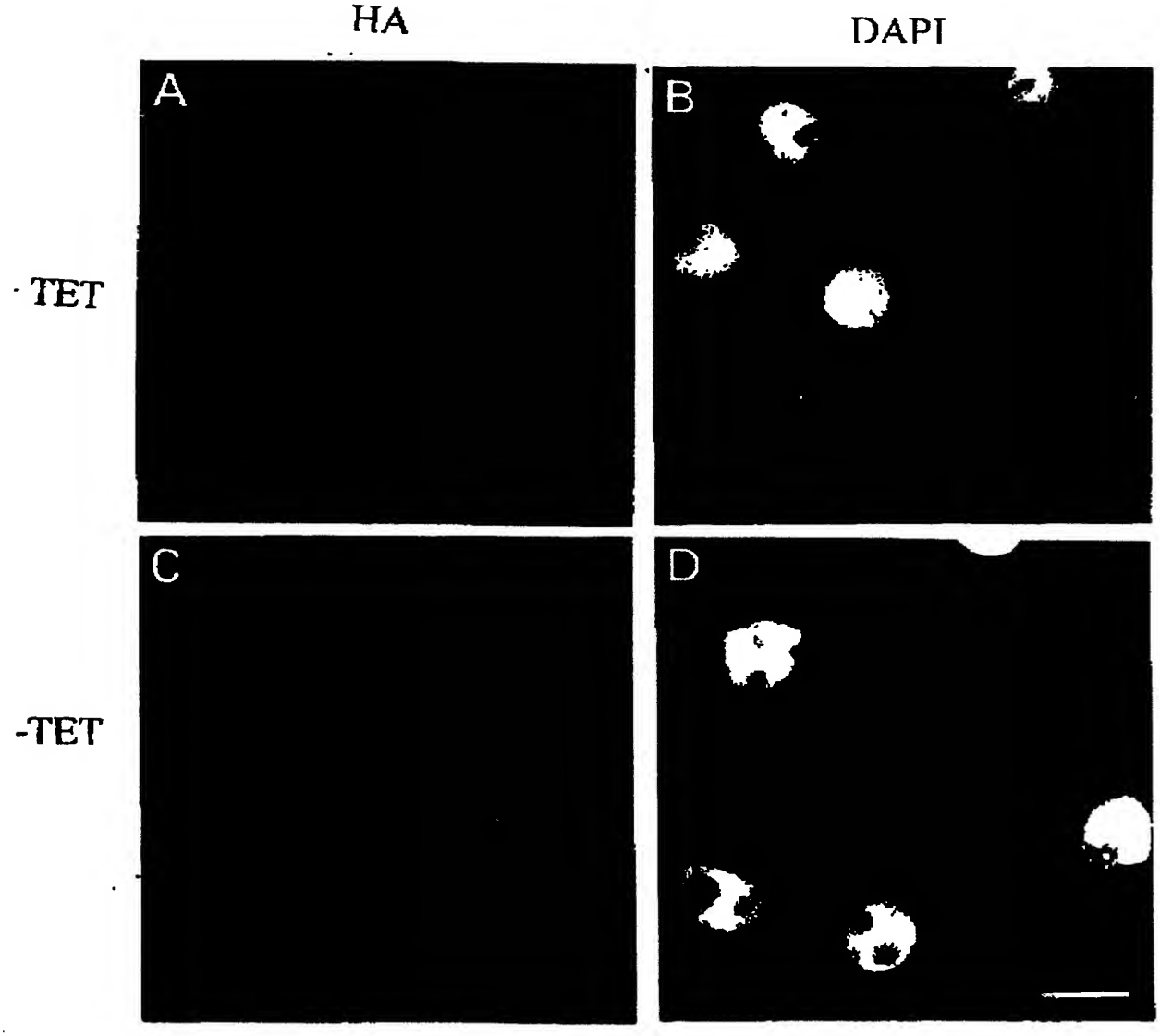


FIGURE 2

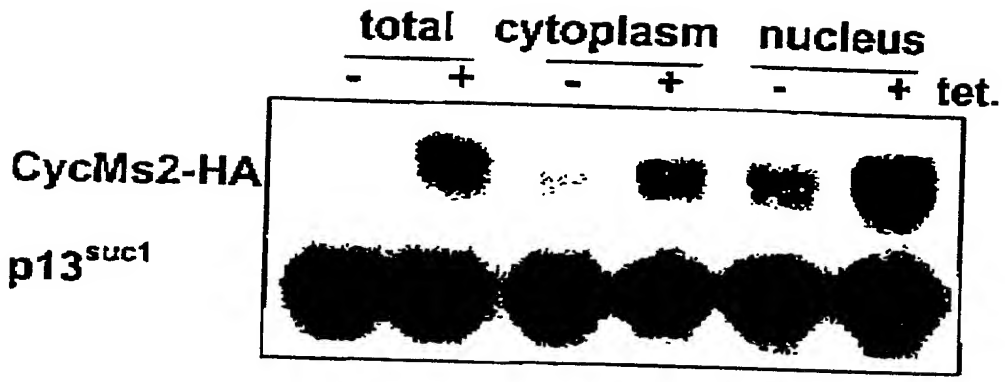
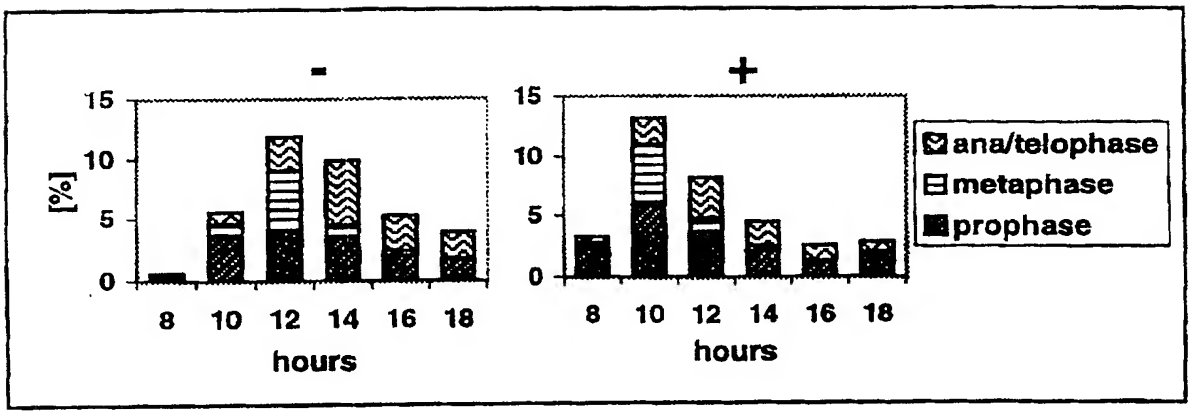


FIGURE 3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+
15
10
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[%]
8 10 12 14 16 18
hours
ana/telophase
metaphase
prophase

FIGURE 4A

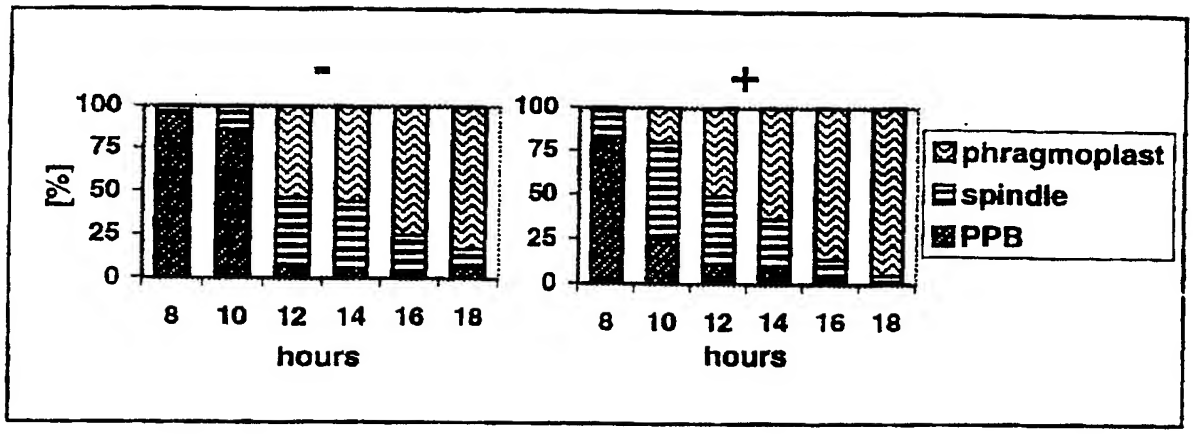


FIGURE 4B

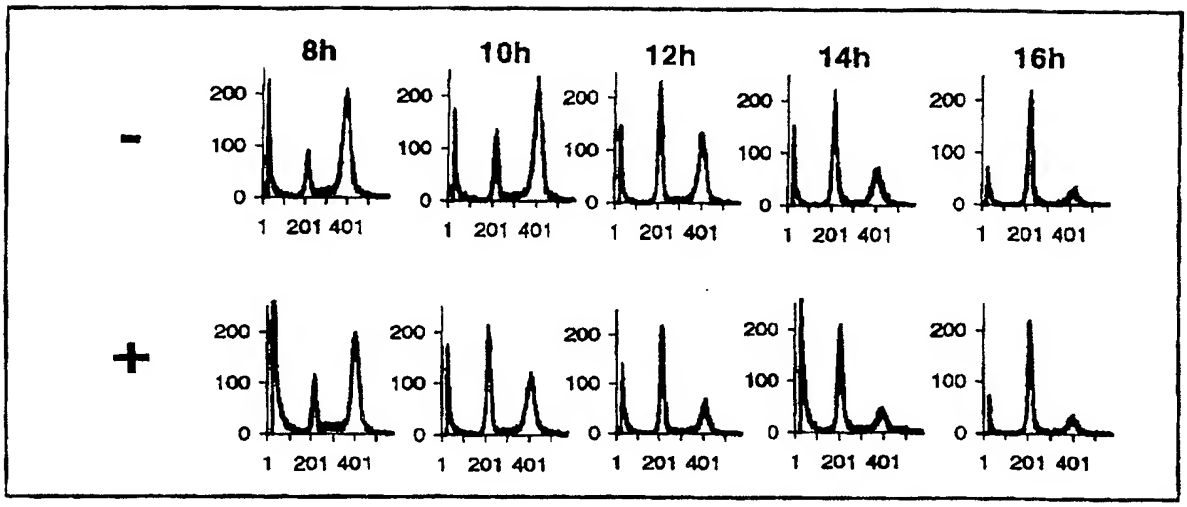


FIGURE 4C

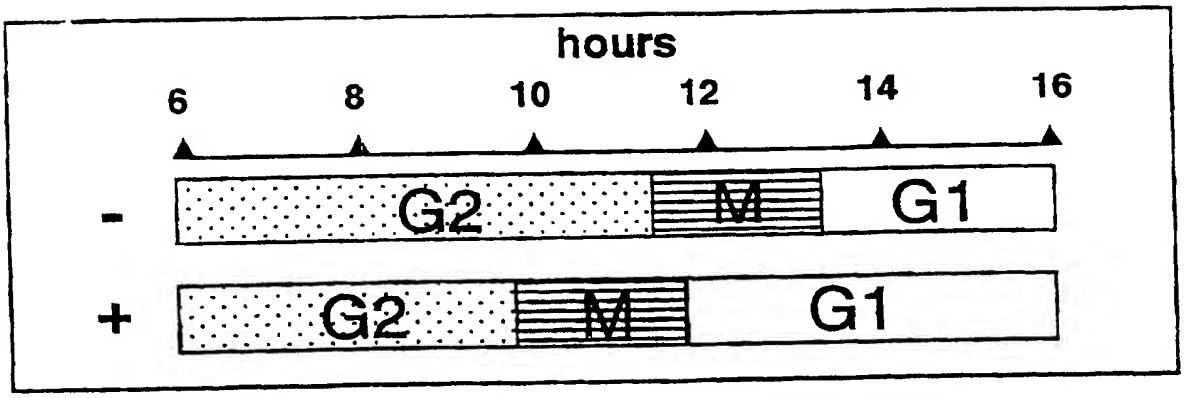
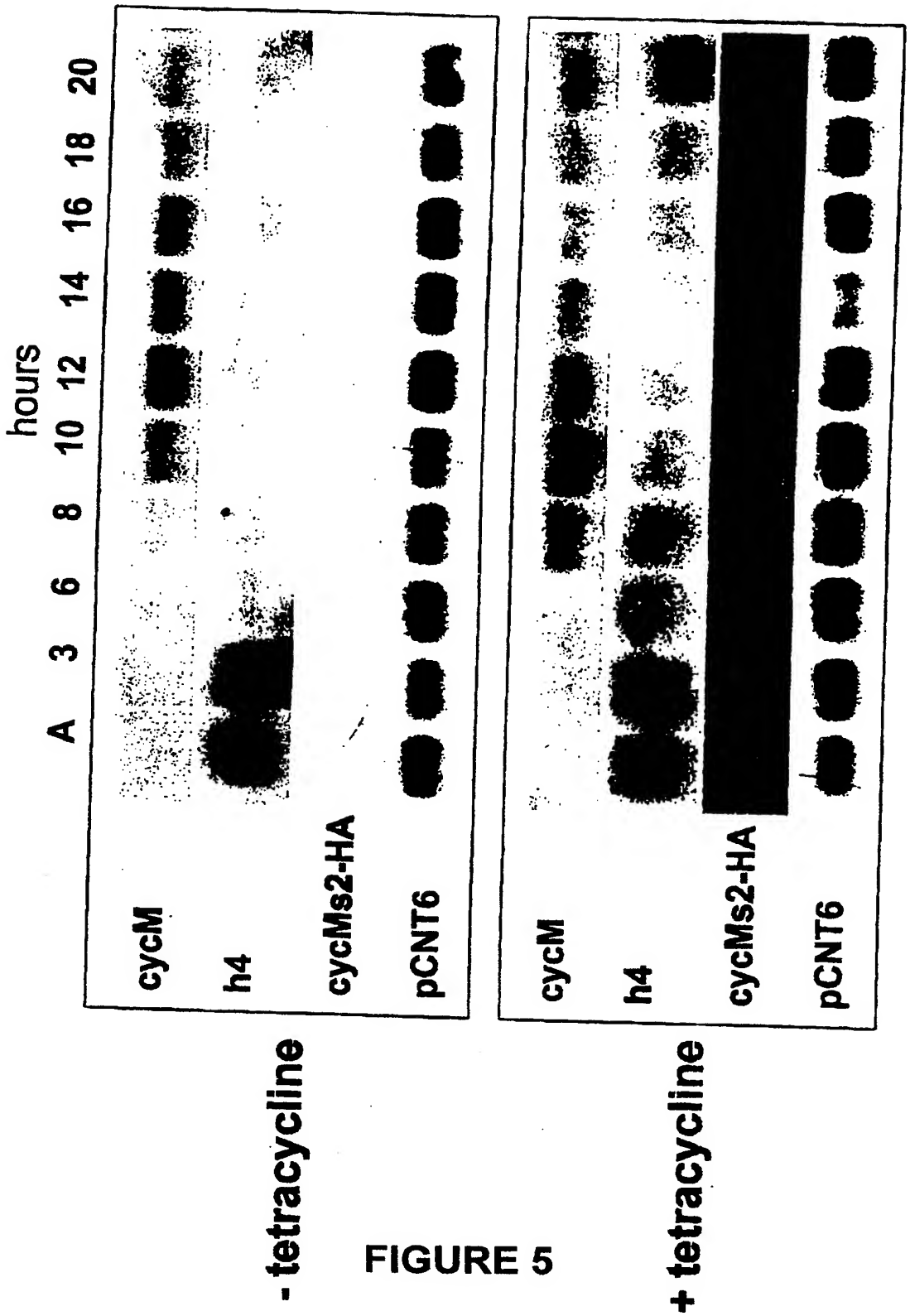


FIGURE 4D


hours
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- tetracycline
cycM
h4
cycMs2-HA
pCNT6
+ tetracycline

FIGURE 5

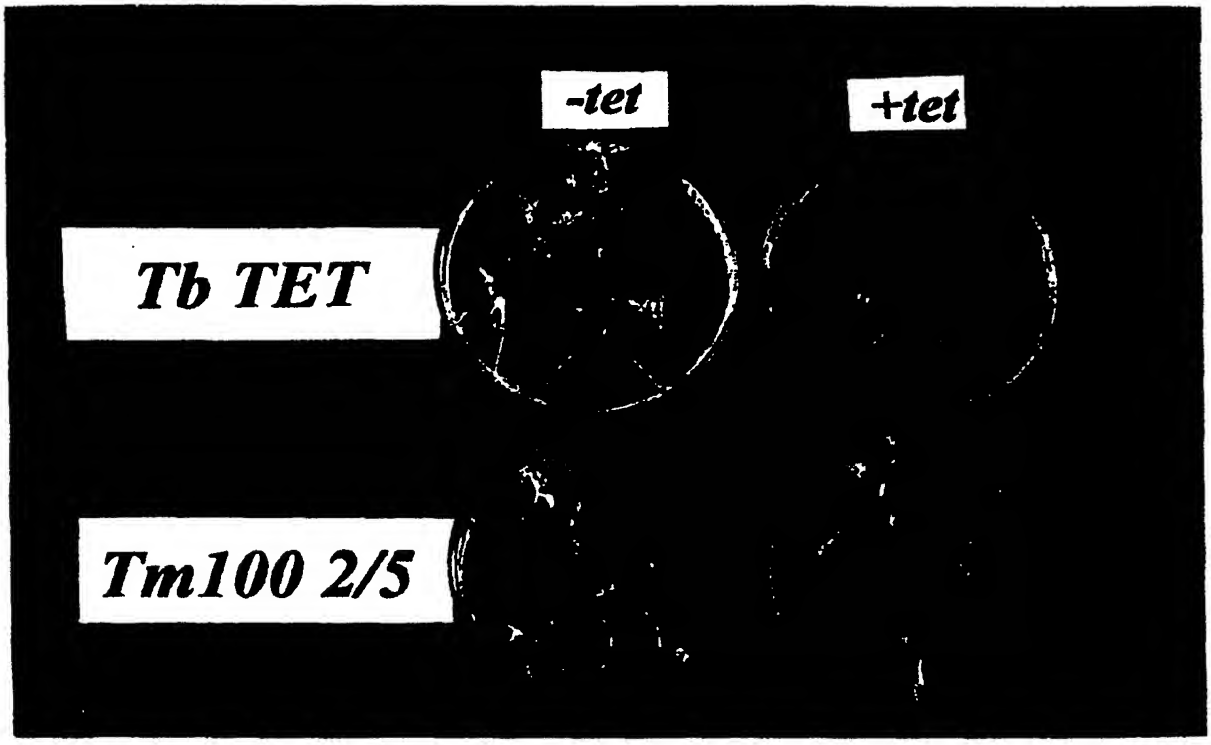
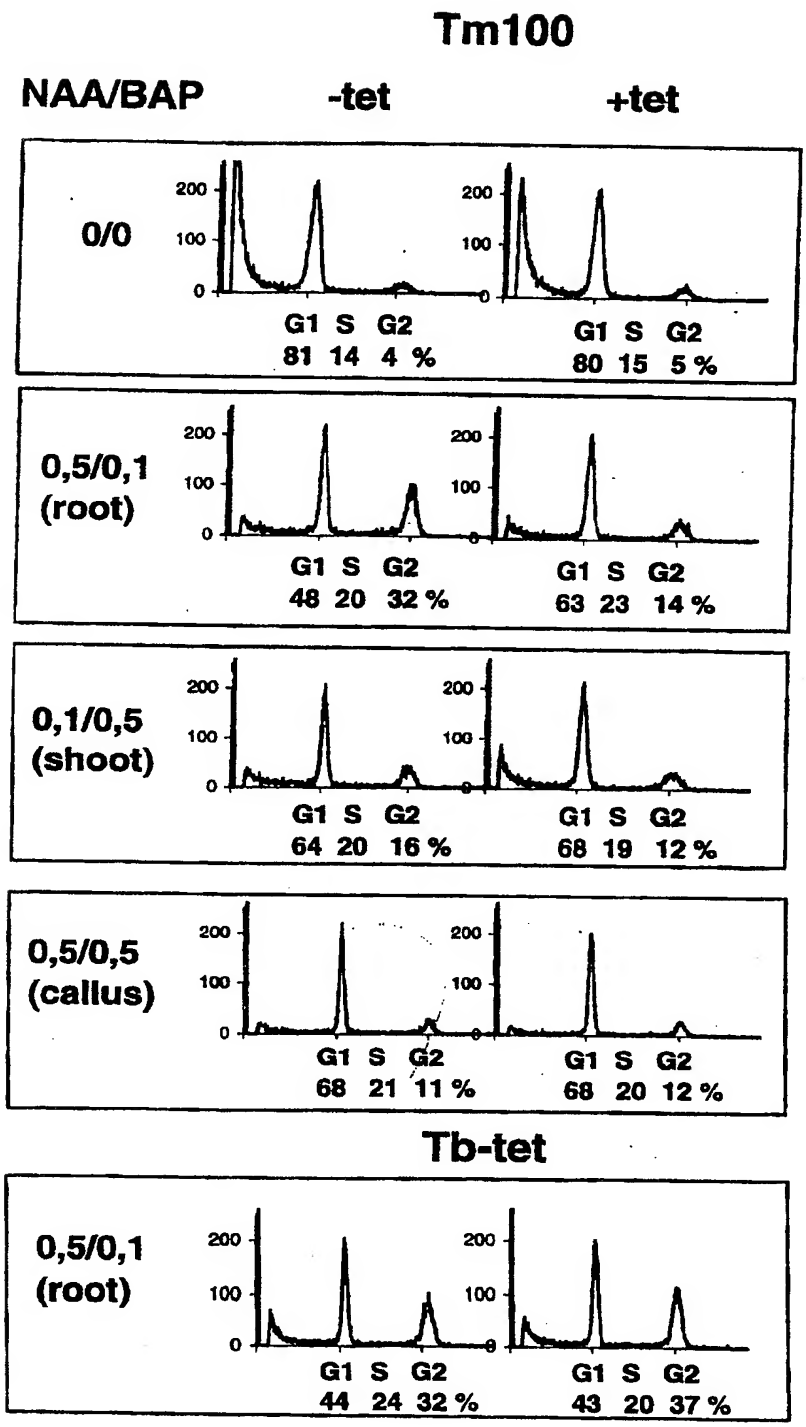


FIGURE 6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NAA/BAP
-tet
+tet
0/0
200
100
0
G1 S G2
81 14 4 %
G1 S G2
80 15 5 %
0,5/0,1
(root)
G1 S G2
48 20 32 %
G1 S G2
63 23 14 %
0,1/0,5
(shoot)
G1 S G2
64 20 16 %
G1 S G2
68 19 12 %
0,5/0,5
(callus)
G1 S G2
68 21 11 %
G1 S G2
68 20 12 %
Tb-tet
0,5/0,1
(root)
G1 S G2
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G1 S G2
43 20 37 %

FIGURE 7

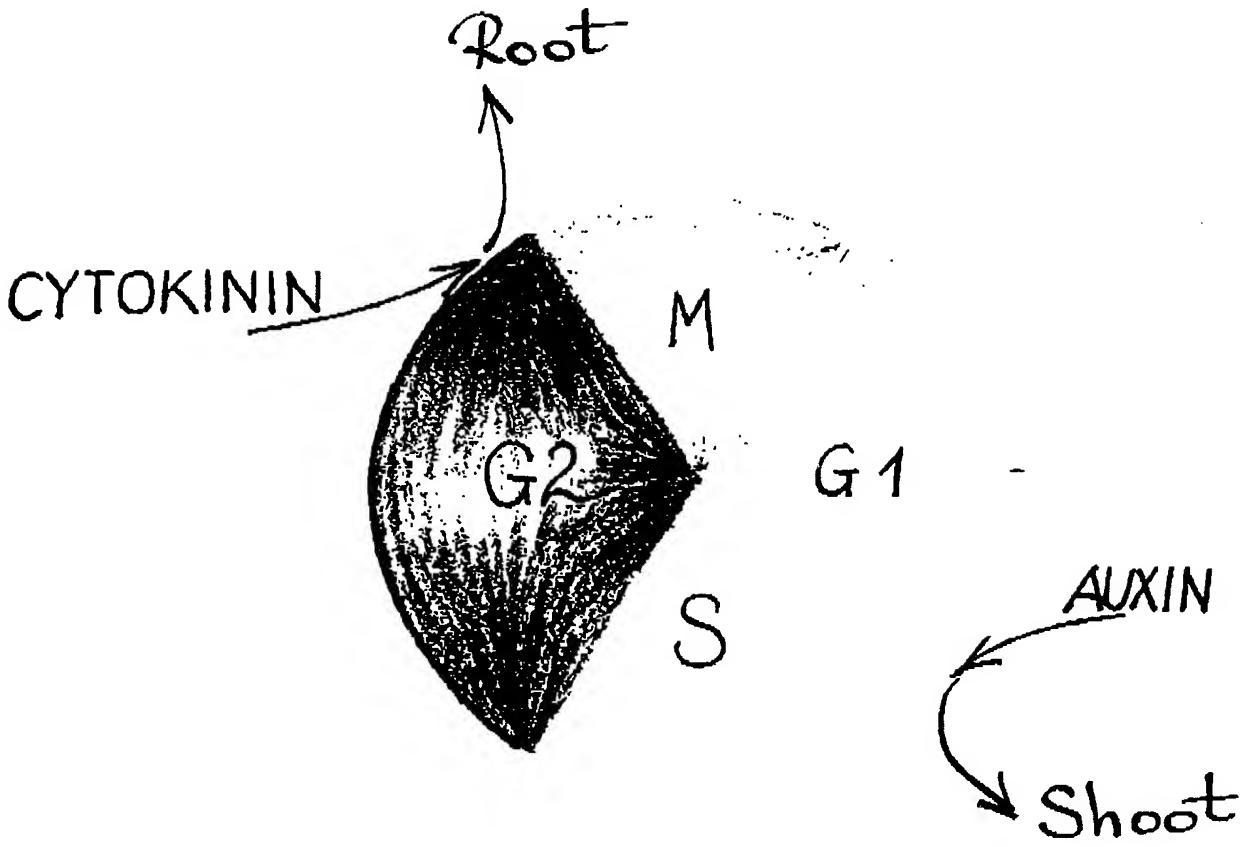
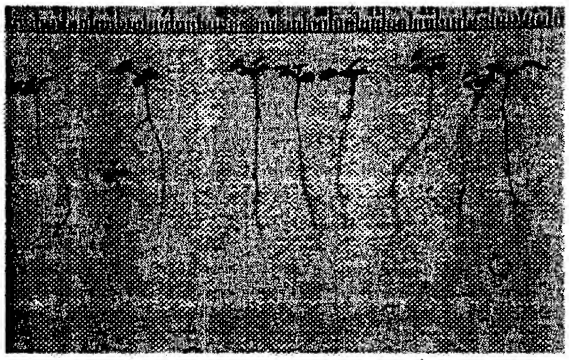


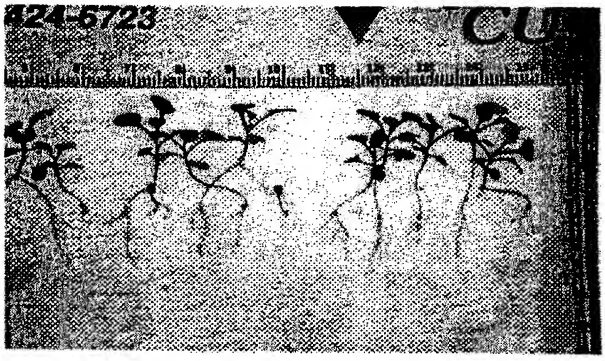
FIGURE 8

TM100 2/5 **Bin Hyg**

18 days



24 days



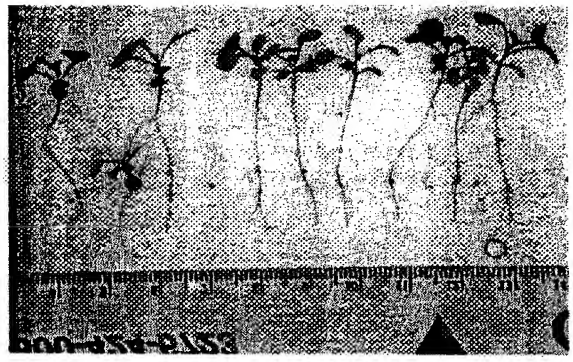


FIGURE 9

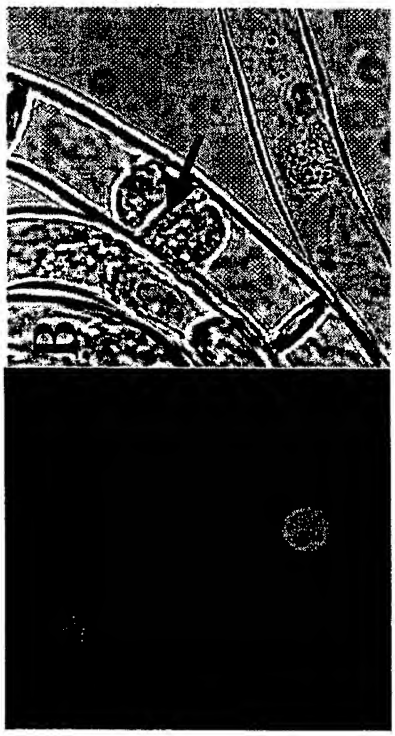


FIGURE 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00137

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 15/29; C12N 15/82; A01H 5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN (CA;WPIDS): cytokins;gibberellin;cyclins

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Physiologia Plantarum vol.93, 365-374 (1995) Francis et al "The Plant Cell cycle". See pp 366; 368; 369	1-67
X	Plant Molecular Biology vol.36, pp601-612 (1998) McKibbin et al "Expression of fission yeast *cdc25* alters the frequency of lateral root formation in transgenic tobacco" See pp602, 609, .	1-67
X	Plant Molecular Biology vol.23, pp445-451 (1993) Bell et al "Tobacco plants transformed with *cdc25*, a mitotic inducer gene from fission yeast".See pp445,446	1-67

☐ Further documents are listed in the continuation of Box C ☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 April 2000	20 APR 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **MADHU K. JOGIA** Telephone No : (02) 6283 2512

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00137

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Planta 206, pp215-224 (1998) Trebin et al "Cell cycle regulation by plant growth regulators..". See pp215, 216, 222	1-67
X	The Plant Journal 11 (2), 181-190 (1997) Sauter, M "Differential expression of a CAK (cdc2-activating kinase)-like protein kinase, cyclins and cdc2 genes from rice during the cell cycle and in response to gibberellin". See p181	1-67
X	Molecular and Cell Biology of the Plant Cell Cycle 9-34 (1993) John , P et al " A p34cdc2-based cell cycle: its significance in monocotyledonous, dicotyledonous and unicellular plants'. See pp16, 30	1-67
X	The Plant Journal 7 (4), 623-632 (1995) Sauter et al "Gibberellin promotes histone H1 kinase activity and the expression of cdc2 and cyclin genes during the induction of rapid growth in deepwater rice internodes". Seep623,	1-67
X	Planta 200, pp2-12 (1996). Zhang et al "Cytokinin controls the cell cycle at mitosis by stimulating the tyrosine dephosphorylation and activation of p34cdc2-like H1 histone kinase" see pp 2, 11	1-67
X	WO 92/09685 (The Australian National University) ;published 11 June 1992; see claim 23	1

INTERNATIONAL SEARCH REPORT
Information on patent family members

PCT/AU00/00137

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	9209685	AU	90462/91	CA	2097286	EP	559729
US	5750862						

END OF ANNEX